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Effects of acute alcohol exposure on glutamate neurotransmission in adolescents and adults: a preclinical study

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To my family

Abstract

The age at which an individual first uses alcohol is a powerful predictor of alcohol dependence later in life. Brain development and maturation continues during adolescence until adulthood in humans. There are continuous morphological and functional alterations within the brain areas involved in emotions, learning, decision-making and reward-motivated behaviors and alcohol exposure during this period may modify the development of these regions, increasing the sensitivity of adolescents to some of alcohol's effects. Thus, adolescents might be or may become more sensitive to the rewarding properties of alcohol than adults. Despite the important role of glutamate neurotransmission for brain development and many other brain functions and despite the fact that ethanol consumption during adolescence may have a detrimental impact on these functions, we know surprisingly little about glutamatergic transmission in the adolescent brain and its modulation by alcohol. Here the acute effects of ethanol on glutamatergic neurotransmission in nucleus accumbens (NAc) were studied using brain slice electrophysiology and glutamate release and dynamics in the prefrontal cortex (PFC) were studied using enzyme-based microelectrode amperometry.

The results of the present thesis reveal several age-dependent differences in glutamatergic neurotransmission in these brain regions. Using extracellular electrophysiology, I found that the inhibitory effect of acute ethanol on glutamatergic transmission (fEPSP/PS) in the NAc was higher in brain slices from adolescent animals compared to brain slices from adults. As previously reported, the mechanism by which acute ethanol inhibits glutamate neurotransmission was found to be presynaptic inhibition of glutamate release and this effect was blocked by GABA receptor antagonists. Moreover, acute ethanol was found to inhibit the induction on long-term potentiation (LTP) in the NAc. In the PFC, glutamate levels in freely moving animals were measured and it was found that basal levels of glutamate were more than three times higher in adolescent animals than in adults. Spontaneous release of glutamate in terms of glutamate transients was higher in the PFC of adolescent rats than in the PFC of adult rats. The transients were inhibited by ethanol in the adolescent animals but they were unaffected in the adults. The data in my thesis thereby confirms previous studies suggesting age-dependent differences in glutamatergic neurotransmission in response to ethanol and extend our knowledge about effects of ethanol on adolescent brain. These age-related differences and differential effects of alcohol on glutamate neurotransmission in adolescent animals may be a contributing factor underlying the increased susceptibility of a young individuals' brain to develop alcoholism or other addictions later in life.

List of publications

I. Mishra D, Chergui K (2011) Ethanol inhibits excitatory neurotransmission in the nucleus accumbens of adolescent mice through $GABA_A$ and $GABA_B$ receptors. Addiction biology 18:605-613.

II. Mishra D, Zhang X, Chergui K (2012) Ethanol disrupts the mechanisms of induction of long-term potentiation in the mouse nucleus accumbens. Alcoholism, clinical and experimental research 36:2117-2125.

III. Mishra D, Harrison NR, Gonzales CB, Schilström B, Konradsson-Geuken Å (2013) Effects of acute ethanol on glutamatergic neurotransmission in prefrontal cortex of awake adolescent and adult rats using enzyme-based microelectrode amperometry. (Manuscript)

Contents

List of abbreviations

AA	L-ascorbic acid
AC	anterior commissure
AcbC	nucleus accumbens core
AMPA	amino-3-hydroxy-5-methyl-4-isoazole propionic acid
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AP	anterior posterior
BLA	basolateral amygdala
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
DAG	diacylglycerol
DARPP	dopamine-and cAMP-regulated phosphoprotein
DHPG	(S)-3,5-Dihydroxyphenylglycine
DV	dorsal ventral
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EAAT	excitaotory amino acid transporter
E-LTP	early-long term potentiation
FAST	fast analytical sensing technology
fEPSP/PS	field excitatory postsynaptic potentials/population spikes
GABA	gamma (γ) aminobutyric acid
GluOx	glutamate oxidase
HFS	high frequency stimulation
iGluR	ionotropic glutamate receptor
IP_3	inositol triphosphate
i.p	intraperitoneal
KA	kainate receptors
LOD	limit of detection

LTD	long term potentiation
L-LTP	late-long term depression
ML	medial lateral
MEA	microelectrode array
mGluR	metabotropic glutamate receptors
m- PD	*m*-phenylenediaminedihydrochloride
MSN	medium spiny neurons
NAc	nucleus accumbens
NMDA	*N*-methyl-D-aspartate
PKA	protein kinase A
PKC	protein kinase C
PFC	prefrontal cortex
PND	postnatal day
S.E.M.	Standard error of mean
TTX	tetrodotoxin
vGLUT	vesicular glutamate transporter
VTA	ventral tegmental area

1. Introduction

1.1 Alcohol use and abuse in society

Alcohol is the most commonly available and abused drug in our societies. Unfortunately, problems related to its consumption such as consequences for an individual's health like liver cirrhosis (Heron, 2004 National vital statistics report volume 56, number 5, www.cdc.gov), nephritis (kidney failure), alcoholic myopathy, ataxia, peripheral neuropathy, gastric ulcers, recurrent abdominal pain, problems with social life including violence, child abuse and maltreatment (The National Centre on Addiction and Substance Abuse report, 1999), problematic family relations (Leonard and Rothbard, 1999) and professional problems like loss of work efficiency (Booth and Feng, 2002) are common as well. In the United States 80,374 people die every year due to excessive alcohol consumption which makes it the third leading cause of death. In the age group of 15 to 24 years, alcohol is the leading cause of death (centre for disease control-CDC, www.cdc.gov). The approximate cost of alcohol abuse is $185 billion annually in terms of health care, loss of productivity, and crime (National Institute on Drug Abuse-NIDA).

In Sweden, with a population of 9.5 million individuals, about 330 000 are estimated to be addicted to alcohol while the number of people who abuse alcohol is around 780 000 (Centralförbundet för alkohol och narkotikaupplysning report 130, 2011) and the calculated costs associated with alcohol use in terms of health care, social services, crime, research policy and prevention, and loss of productivity were estimated to be over 45 billion Swedish crowns in 2011 (Allbeck et al., 2012). Thus, in addition to problems at the individual level, alcohol consumption is also a financial burden on society.

Epidemiological studies have repeatedly come to the conclusion that age at first alcohol use is a powerful predictor of lifetime alcohol dependence (DeWit et al., 2000; Grant et al., 2001; Hingson et al., 2006a and b). It is, therefore, important to work both preventatively in order to protect the young people from developing alcoholism and also to find better treatments for those who have become addicted. This thesis is focused on understanding age-dependent neurobiological effects of alcohol and hopefully, the obtained knowledge from this field of neuroscience and other research areas will be useful in understanding the mechanisms by which alcohol affects the young brain and develop or improve treatments based on these understandings.

1.2 Alcohol abuse, dependence and addiction

The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 1993), characterizes abuse and dependence separately. According to DSM-IV**, alcohol abuse** is defined as the

maladaptive recurrent alcohol use leading to clinically significant impairment or distress as manifested by one (or more) of the following, occurring within a 12-month period: 1) recurrent alcohol use despite failures to fulfil major obligations at work, school or home 2) recurrent alcohol use in physically hazardous situations 3) recurrent alcohol use-related legal problems 4) continued alcohol use despite persistent or recurrent social problems caused or exacerbated by the effects of alcohol.

Alcohol dependence, which is the more severe diagnosis, is characterized by or defined as a maladaptive pattern of alcohol use leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring any time within the same 12-month period: 1) tolerance, defined as markedly increased amounts of alcohol consumption to achieve intoxication or the desired effect or markedly diminished effect with the use of same amount of alcohol 2) The characteristic withdrawal syndrome for alcohol; or drinking (or using a closely related substance) to relieve or avoid withdrawal symptoms 3) alcohol is consumed in larger amounts or over a longer period than was intended 4) a persistent desire or unsuccessful efforts to cut down or control use of alcohol 5) a great deal of time is spent in activities necessary to obtain alcohol, using alcohol or recovering from its effect 6) important social, occupational, or recreational activities are given up or reduced because of the continued alcohol use 7) alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the alcohol.

Recently, DSM-V has been published and in this updated version alcohol abuse and dependence has been merged together into a single disorder measured on a continuum from mild to severe. As mentioned above, a diagnosis of alcohol abuse previously required only one symptom, but, mild substance use disorder in DSM-V requires two to three symptoms from a list of 11. The criterion regarding alcohol-related legal problems has been eliminated because cultural differences made the criterion difficult to apply internationally. In addition, the DSM-V has added craving as a new criterion which can be defined as a strong urge or desire to consume a substance even after long periods of abstinence.

The currently available drugs for alcohol addiction like naltrexone (O´Malley et al., 1992; Volpicelli et al., 1992), acamprosate (Sass et al., 1996), busiprone (Kranzler et al., 1994) have been helpful in alleviating alcohol consumption, however, the effect of these drugs seem to be limited and varying in different populations, as in case of naltrexone, where alcoholics with a family history of alcoholism show better reductions in alcohol consumption compared to alcoholics with no family history of alcoholism (Anton et al., 2008; Gelernter et al., 2007: Krishnan-Sarin et al., 2007). In case of acamprosate, the effectiveness of the drug in maintaining abstinence has not been consistent, with European studies showing it to be effective, while some American findings suggest no or modest effects (Mann et al., 2004; Anton et al., 2006; Mason et al., 2006). Despite psychological and pharmacological treatment in some studies the relapse to alcohol consumption was found in 50% of patients within 12 weeks of treatment with naltrexone (O´Malley et al., 1992) and in 60% within 1 to 12 weeks following acamprosate treatment. With a combination of naltrexone and acamprosate treatment the

relapse occurred in 25% of all the patients (Kiefer et al., 2003). Clearly, better treatments are needed but the problem is that, despite research progress in the last couple of decades, the neurobiology of addiction is still far from clear and we need better understanding of how alcohol affects the brain.

A variety of external and internal factors that initiate, sustain and direct behavior can be referred to as motivational cues. Natural rewards such as food and water, which are required for physical well-being and survival (hunger and thirst), provide a satiating or positive feeling once consumed and thereby provide positive reinforcement of the preceding behavior. Drugs of abuse share similar properties with natural rewards initially, but consumption of addictive drugs is more positively reinforcing than natural rewards. The idea of positive reinforcement by drugs of abuse has been suggested as a primary initial factor driving the development of drug dependence. Repeated use of drugs of abuse may lead to a decrease in their reinforcing effect which in turn may lead to a compensatory increase in the amount of drug consumed. Long-term drug use can in fact result in negative psychological and physiological effects if consumption is suspended, thus inducing continued drug use as a means to evade the aversive consequences of drug withdrawal i.e. negative reinforcement. This theory of positive and negative reinforcement provides a general idea about the initiation and persistence of drug use (see Cami and Farre, 2003, Feltenstein and See, 2008), although, it is still unable to explain many other aspects of drug dependence like relapse of drug seeking and drug-taking following a prolonged period of abstinence. Other theories of addiction have been proposed, for example, the theory of pathological shift of hedonic-set points in the drug user, requiring high amounts of drug to attain the same rewarding feeling as with first exposure (Koob and Le Moal, 1997). The theory of incentive sensitization (Robinson and Berridge, 1993; Robinson and Berridge, 2001) proposes that drug abuse induces modifications in number of neural systems, especially in brain areas involved in incentive motivation and reward for natural reinforcers like food, water and sex. Thereby, repeated drug use leads to a shift from drug-liking to drug-wanting which in turn leads to compulsive or habitual patterns of drug-seeking behavior.

Several other theories specifically explaining the course and perseverance of drug addiction offer to explain the persistent nature of addictive behaviors that include a shift of neural control over drug-taking behavior from the prefrontal cortex (PFC) to nucleus accumbens (NAc) and eventually to dorsal striatum possibly mediated via neuroplasticity changes in these regions (see Everitt and Robbins, 2005; Koob and Volkow, 2010). Changes in prefrontal cortical functions are suggested to lead to loss of behavioral control and decision-making ability and thereby increase the risk of relapse and decrease the ability to terminate potentially harmful behaviors (Goldstein and Volkow, 2002; 2011). The maladaptive learning theory suggests that because addictive drugs induce larger dopamine release, an abnormal stimulus-response behavior is learned and makes drug-related stimuli more salient than stimuli related to natural rewards (see Di Chiara, 1999). Each of these theories covers or explains unique aspects of addiction as a process and there is often significant overlap among these different perspectives.

Although, it seems difficult to define addiction in humans by just one of the above theories, there are many aspects of the neurobiological substrates underlying addiction that are common across species that can be studied using animal models. For example, all drugs of abuse, lead to an excessive release of the neurotransmitter dopamine in the NAc (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988; Volkow et al., 2004). The dopaminergic pathways have been extensively implicated for their involvement in the rewarding properties of both natural stimuli and addictive drugs and dopamine release appears necessary for reward (Di Chiara and Imperato, 1988: Hernandez and Hoebel, 1988). Moreover, various manipulations of the dopaminergic systems alter drug self-administration in animals (Roberts et al., 1980; Roberts and Koob, 1982; Hubner and Koob, 1990). Therefore, the large release of dopamine is assumed to provide positive reinforcement for drug self-administration and possibly initiating the addiction process.

Nevertheless, certain manipulations of dopaminergic neurotransmission like infusion of a dopamine receptor antagonist or selective lesions of dopamine neurons in the NAc using 6-hydroxydopamine which can block psychostimulant self-administration (Lyness et al., 1979; Belin and Everitt, 2008), however, failed to affect alcohol self-administration (Rassnick et al., 1993), suggesting dopamine-independent mechanisms maintaining alcohol self-administration. Moreover, following repeated drug use (alcohol) other brain regions like the PFC have been shown to be dysfunctional as well. Normally, the PFC mediates the executive functions of the brain, including inhibitory control of potentially harmful activities. Thus, loss of PFC functions, as seen in alcohol addicts, results in a compromised ability to control craving and thereby, relapse to alcohol consumption (Goldstein and Volkow, 2011). Once addicted an individual develops such a strong motivation to consume the drug that he/she is unable to inhibit or control the alcohol consumption despite awareness of the negative consequences associated with alcohol consumption. Overall, alcohol-induced increases in dopamine in the NAc are thought to underlie the reinforcing responses to alcohol consumption (Weiss et al., 1993) and could be termed as an initial stage of addiction via **positive reinforcement**. With repeated alcohol consumption there is a series of adaptations resulting in **tolerance** which may manifest as **withdrawal** symptoms. At the same time there may be loss of control (dysfunctional PFC) over alcohol-intake resulting in compulsive alcohol consumption which characterizes **addiction**.

1.3 Alcohol consumption and the developing brain: why age matters?

As mentioned above, a number of epidemiological studies have shown that age at first alcohol use predicts the risk of lifetime alcohol dependence (DeWit et al., 2000; Hingson et al., 2006a and 2006b). Individuals who report consumption of alcohol before the age of 15 are four times more likely to also report meeting the criteria for alcohol dependence later in their lives (Grant and Dawson, 1998; Dawson et al., 2008; Hicks et al., 2010). Human brain imaging studies reveal that alcohol addicts show depletion in cortical grey matter [consisting of neuronal cell bodies, neuropil, glial cells (astroglia and oligodendrocytes) and capillaries] (Pfefferbaum et al., 1998; 2001; Fein et al., 2002) when compared to those of healthy volunteers. Many brain

regions continue to mature until adulthood; therefore, alcohol consumption during adolescence may severely affect brain development and function. Indeed, adolescent brain scans reveal that alcohol affects the health of white matter in the brain with a less efficient thinking process as a possible outcome (McQueeny et al., 2009). McQueeny and colleagues (2009) utilized diffusion imaging technique in which a high fractional anisotropy index is a measure of hindrance of water diffusion, which in turn is related to the presence of oriented structures. Fractional anisotropy index has been used in studies to evaluate the influence of alcohol use on white matter integrity (Rosenbloom et al., 2003) and it has been shown to be reduced in the corpus callosum (Schulte et al., 2005, see Pfefferbaum and Sullivan, 2005 and references therein), right frontal lobe (Harris et al., 2008), and global white matter in adults (McQueeny et al., 2009). Supporting the idea that disturbances in white matter integrity may lead to inefficient information processing, a recent longitudinal brain imaging study showed that adolescents who developed heavy alcohol drinking habits during a three-year period performed worse than those who were still non-users on tasks requiring working memory (Squeglia et al. 2012).

So far, ethical boundaries have limited our knowledge of the neurobiological differences between human adolescents and adults, but animal models have been helpful in examining age-dependent differential effects of ethanol. Thus, studies in rats have demonstrated several age-dependent differences in response to ethanol (see Spear, 2013). For example, when given free access to ethanol, adolescent rats consumed more (Doremus et al., 2005). Adolescent rats also show reduced taste aversion (Vetter-O'Hagen et al., 2009) and reduced sensitivity to motor impairing effect of ethanol when compared to adults (Silveri and Spear., 2001). Ethanol is also known to interfere with learning and memory and has been shown to induce greater impairment in a hippocampus-regulated spatial memory task in adolescent animals compared to adults (Markwiese et al., 1998). Moreover, adolescent animals are more sensitive to ethanol-induced neurodegeneration compared to adults (Crews et al., 2000, 2004; Pascual et al. 2007). At the synaptic level, ethanol-mediated inhibition of glutamatergic neurotransmission is greater in adolescents than in adults (White and Swartzwelder, 2005).

1.4 Glutamate and glutamatergic neurotransmission

The excitatory neurotransmitter glutamate mediates almost 70% of neurotransmission within the central nervous system (see Danbolt, 2001 and references therein) and is thought to be central in regulating neuroadaptational processes in the brain (see Selemon, 2013). Glutamate is packed into synaptic vesicles in the presynaptic terminal by vesicular glutamate transporters (vGluTs) using a proton gradient generated using the energy from hydrolysis of adenosine triphosphate (ATP). So far, three different vGluTs have been identified (vGluT1–3) (Danbolt, 2001; Shigeri et al., 2004). Following release, glutamate is cleared from the extracellular space by a family of sodium (Na^+)-dependent excitatory amino acid transporters (EAATs) and identified distinctly as EAAT1 to 5. EAAT1–3, are also known as GLAST, GLT-1, and EAAC1, respectively. EAAT2 and EAAT5 are expressed in presynaptic terminals, EAAC1 and EAAT4 are present postsynaptically, and GLAST and GLYT-1 are expressed in glial cells. The EAATs

prevent excessive accumulation of extracellular glutamate, which can result in excitotoxicity. The major part of glutamate uptake is mediated by GLAST and GLT-1 and once inside the glial cells, glutamate is converted to glutamine by glutamine synthetase which is then secreted from the glia and taken up by the presynaptic terminal for conversion back to glutamate by glutaminase. Glutamate can also be transported from the glial cells to the extracellular environment by the cysteine-glutamate exchanger (X_c) (Melendez et al., 2005; Baker et al., 2002).

Glutamate released in the extracellular space can bind to two different classes of glutamate receptors (iGluRs); the ionotropic receptors (*N*-methyl-D-aspartate (NMDA) receptor, the alpha-amino- 3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, and the kainic acid (kainate, KA) receptor) and the metabotropic glutamate receptors (mGluR group I and II).

1.4.1 Ionotropic glutamate receptors

NMDA receptors are heterotetrameric protein complexes that form ligand-gated ion channels composed of two NR1 subunits (at least eight splice variants exist) and a combination of two NR2A-D and NR3A or 3B subunits (see Dingeldine et al., 1999; Stephenson, 2006 and references therein). In addition to glutamate which is an endogenous agonist, the stimulation of NMDA receptors also requires a co-agonist such as D-serine, glycine or D-alanine. The NR2 subunit contains the glutamate binding domain and the NR1 subunit contains the glycine binding domain. During hyperpolarized states, the NMDA receptor channel pore is blocked by Mg^{2+} ions, but once adequate membrane depolarization has been established, the Mg^{2+} block is removed, allowing the influx of Ca^{2+} ions causing a large depolarization. Notably, NMDARs containing the subunits NR2C, NR2D and NR3 have a reduced Mg^{2+} inhibition and require lower depolarization to open the NMDA receptor channel pore (Momiyama et al., 1996; Chatterton et al., 2002; see Cull-Candy and Leszkiewicz, 2004). The NMDA receptor is permeable to K^+ and Na^+ ions as well. Activation of the NMDA receptor is modulated by polyamines and is inhibited by Zn^{2+}. The subunit composition of NMDA receptors is ontogenetically regulated with predominant NR2B subunits initially and switched to NR2A dominance with development (Sheng et al., 1994; Quinlan et al., 1999). Apart from the postsynaptic nerve terminals, NMDA receptors have also been shown to be expressed on presynaptic terminals (Casado et al., 2000) as well as on glial cells including microglia, astrocytes, and oligodendrocytes (Paoletti and Neyton, 2007).

AMPA receptors are ligand-gated ion channels which are heterotetrameric protein complexes composed of subunits termed GluR1–4 (also termed GluRA-D). Each GluR subunit contains a binding site for glutamate (see Dingledine et al., 1999 and references therein). Once activated, AMPA receptors become permeable to various cations including Ca^{2+}, Na^+ and K^+. The majority of AMPA receptors in the brain contain GluR2 subunits, which render the channel impermeable to Ca^{2+}. AMPA receptor function can also be blocked by polyamines-mediated ion channel block (Bowie and Mayer 1995; Bowie et al., 1998).

KA receptors are tetrameric protein complexes forming ligand-gated ion channels composed of different subunits. These subunits are named GluR5-7. KA receptors are permeable to Na^+ and K^+ ions and activated upon Na^+ influx. KA receptors, along with postsynaptic sites, are also localized on presynaptic terminals where they can modulate neurotransmitter release (see, Huettner, 2003 and references therein).

1.4.2 Metabotropic glutamate receptors

As mentioned above, along with ionotropic receptors, glutamate can also bind to metabotropic glutamate receptors (mGluRs).The mGluRs are located throughout the extraceulllar space with perisynaptic location on glial cells, on the presynaptic terminals and on postsynaptical terminals. Structurally mGluRs are seven transmembrane domain-containing G-protein coupled receptors and are thought to be mediating slower, modulatory neurotransmission compared to ionotropic receptors (see Conn and Pin, 1997 and references therein; Cooper et al., 2003). mGluRs are divided into two distinct groups:

Group I mGluRs are further divided into mGluR1 and mGluR5. Group I mGluRs activate the $G\alpha_q$ class of G-proteins, stimulating phospholipase C, resulting in phosphoinositol hydrolysis and formation of lipid signalling intermediates such as inositol triphosphate (IP_3) and diacylglycerol (DAG), which in turn can activate various intracellular messengers including protein kinase C (PKC). Activation of Group I mGluRs also activates calcium release from IP_3 receptor-mediated intracellular stores, which in turn could activate other intracellular messengers such as Ca^{2+}-calmodulin dependent kinase II (CaMKII). Group I mGluRs, particularly mGluR5, are positively coupled to NMDA receptor function via PKC, and are structurally linked to these receptors as well as to IP_3-gated intracellular Ca^{2+}-channels via the Homer family of proteins. Group I mGluRs are located on postsynaptic cells (mGluR5 on glial cells as well) and seldom present on presynaptic terminals.

Group II (mGluR2 and mGluR3) and **Group III** (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs activate the $G\alpha_i$ class of G-proteins and are negatively coupled to adenylyl cyclase (AC) activity. Stimulation of these receptors results in a decreased intracellular levels of cyclic adenosine monophosphate (cAMP). Group II and Group III mGluRs are located on presynaptic terminals, particularly mGluR2 and mGluR3, and mediate the classical inhibitory autoreceptor mechanism that suppresses glutamate release. mGluR3 and mGluR5 are also localized on glial cells such as astrocytes. All these receptors play a significant role in generating and /or modulating action potentials and therefore, regulating neurotransmission (see Hermans and Challiss, 2001; Coutinho and Knopfel, 2002; and references therein, Mudo et al., 2007)

In addition to its role in synaptic transmission, activation of NMDA receptors can trigger various intracellular signalling cascades including protein kinase A (PKA), mitogen-activated protein kinase, and extracellular signal-regulated kinase. Activation of additional signalling molecules, like PKC, can also be achieved by stimulation of mGluRs. Together, the simultaneous activation of NMDA and mGluRs activates a host of intracellular signalling pathways that result in protein phosphorylation of ion channels, other kinases, and transcription factors that eventually lead to

the molecular events underlying synaptic plasticity. These events can lead to branching and development of synapses via de novo protein synthesis, changes in gene expression and cytoskeletal remodelling. Convincingly, glutamate-mediated synaptic plasticity has been shown to induce changes in iGluR subunit trafficking such as insertion of AMPA receptors into postsynaptic plasma membrane. It is believed that iGluRs, especially NMDA and AMPA are necessary for the induction of many forms of synaptic plasticity including long-term potentiation (LTP), which is considered to be the experimental equivalent of learning and memory, inducing similar changes at cellular and molecular levels in the brain (see Malenka and Bear, 2004).

1.5 Learning and memory

Learning is the process by which we acquire knowledge about our surroundings, while memory is the process by which that knowledge is encoded, stored and later retrieved. During high frequency stimulation (HFS) large amounts of glutamate is released which in turn activates glutamate receptors. Activation of ionotropic receptors depolarizes the cell and removes the voltage-dependent Mg^{2+}-blockade of the NMDA receptors resulting in Ca^{2+} influx leading to even stronger depolarization. Increased intracellular Ca^{2+} activates a variety of Ca^{2+}-dependent enzymes (kinases) and triggers the early, protein synthesis–independent phase of LTP also known as early-LTP (E-LTP). Following the induction of E-LTP, a synapse may undergo further strengthening and develop so called late phase LTP. This late phase or L-LTP has been proposed to be the mechanism by which long-term memory is stored (see Malenka and Bear, 2004; Lynch, 2004; Kandel, 2000). It is dependent on the synergistic activation of target cell, through various inputs like excitatory glutamatergic inputs and modulatory dopaminergic inputs. Along with repeated and strong glutamate stimulation, dopamine triggers the formation of cAMP via activation of the dopamine D_1 receptors which induces the cAMP-PKA-CREB (cAMP-response-elements binding protein) pathway responsible for triggering the formation of new membrane proteins and facilitating the branching of the synapse. Following LTP the surface expression levels of AMPA has been shown to be increased (see Bear, 2003 and references there in) and this increased expression along with new protein synthesis triggers further branching and strengthening of synapses.

Recent behavioral experiments, assessing the physiological correlates for learning, report LTP-like increases in hippocampal synaptic activity in mice that were trained in a hippocampus-dependent procedure known as trace eye blink conditioning (Gruart et al., 2006) and support the relation between learning and neuronal activity. Moreover, both learning (water maze test) and plasticity were shown to be affected in the presence of NMDA receptor antagonist (AP-5) which indicated that this glutamate receptor subtype controls the induction of LTP (Morris et al., 1989; Harris et al., 1984; see Morris, 2013 and references therein). By using knockout models where the animals lack the NR1 subunit of NMDA receptors specifically located on pyramidal cells in the CA1 region of hippocampus, Tsien and colleagues (1996) have shown that HFS failed to induce LTP in the mutant mice while LTP could be readily induced in the wild type animals. Moreover, after training, the wild type animals were able to learn and find the location

of a submerged platform in the water maze test faster compared to the mutant mice. Furthermore, the knock out animals were slower to learn initially, and did not remember where the platform was and spent equal amount of time (25%) in all quadrants of the water maze cylindrical tank.

The above mentioned studies were based on tasks requiring learning and potentiation of synapses in the hippocampus, but the processes regulating reward related behaviors are still being examined and have been difficult to pin-down. Experiments performed by Schultz and colleagues support the basis of reward based learning. In these experiments, single dopaminergic neurons were recorded in alert monkeys while they received appetitive rewards like fruit juice. The neurons were transiently potentiated upon receiving the juice while other stimuli (aversive) like air puffs to the hand did not cause the transient activation of neurons. After repeated pairing of visual (light) and auditory (tone) cues followed by reward, the timing of activation of neurons changed from firing just after the reward was delivered to the exact time when the cue was presented. This change in neuronal activation resembles the transfer of the animals appetitive-reward mediated potentiation reaction initially, to a learned response i.e. from unconditioned stimulus to conditioned stimulus-learning. In another experiment a naïve monkey was required to press a lever, before appearance of a light, to receive an appetitive reward. After several days of training, the animals learned to reach the lever as soon as the light was turned on and this behavioral change correlated with the firing pattern of dopaminergic neurons (Mirenowicz and Schultz, 1996, see Schultz et al., 1997). These studies suggest an intricate relationship between reward and learning.

1.6 Brain regions mediating reward-related behaviors

The brain's reward circuitry was first discovered by Olds and Milner in the early 1950s. They showed that animals recurrently returned to an area in which they had received mild electrical stimulation of subcortical structures anatomically associated with the medial forebrain bundle. The animals would unfailingly perform tasks (e.g. lever pressing) in order to receive brain stimulation (Olds and Milner 1954, Olds, 1956; Olds, 1958; see Milner, 1991 and references therein). Extensive mapping studies of the rodent brain, confirmed that a large majority of the brain sites supporting brain stimulation reward are associated with the nuclei of origin, tracts and terminal region of the medial forebrain bundle i.e. the hypothalamus and related structures.

Gallistel and colleagues, (1981), using electrophysiology, determined that the neural substrate supporting electrical brain stimulation reward was the myelinated descending neural fiber system of the medial forebrain bundle. This system was shown to originate in the deep subcortical limbic region anterior to the hypothalamus and preoptic area, descending to the ventral tegmental area of the midbrain via the medial forebrain bundle, and then ascending, again via the medial forebrain bundle, to a specific group of forebrain limbic regions including the NAc, olfactory tubercle and frontal cortex. Following the leads from the above experiments, direct stimulation of the NAc using different drugs of abuse or electrical stimulations have established that this region is a key regulator in the generation of reward-motivated behaviors

and changes in neural plasticity within this region can underlie and explain many addiction-related behaviors (see Gardner, 2011 and references therein).

1.6.1 Nucleus accumbens

The NAc consists of gamma (γ) aminobutyric acid (GABA)-ergic medium spiny neurons (MSNs) accounting to almost 95% of the neuronal population in the NAc with the rest 5% being the local circuit cholinergic and GABAergic interneurons (Kawaguchi, 1993a). *In vivo* electrophysiological recordings of medium spiny neurons show a representative pattern of spontaneous activity consisting of long periods of silence (down-state) and by brief episodes of firing (up-state) (Wilson and Kawaguchi 1996). Under *in vitro* conditions, MSNs display this two membrane potential states: a hyperpolarized down-state (-60 to -90 mV), when MSNs are in their resting stage, which is attributable to the activation of a strong and rapidly activating inwardly rectifying potassium-selective current known to be present in these cells (Nisenbaum and Wilson, 1995). Once depolarized, the MSNs display a membrane potential of -60 to -40 mV. This up-state or depolarized state is dependent on the stimulations coming from a large number of cortical and thalamic fibres in the striatum. During the up-state the membrane potential may reach the spike threshold which triggers a train of spikes in the MSN (Calabresi et al., 1990a and b; Wilson, 1993). Disturbances in these two-state potentials have been proposed as major neuroadaptations underlying addiction (Mu et al., 2010; Kim et al., 2011).

NAc receives glutamatergic inputs and acts as an interface between limbic and movement controlling brain structures (Mogenson et al., 1980). It integrates the limbic (from ventral hippocampus) and cortical (from medial PFC) information and projects to the ventral pallidum which sequentially sends feedback projections to the PFC via medio-dorsal nucleus of the thalamus (Groenewegen et al., 1999). Therefore, the NAc plays an important role in the integration of contextual learning and executive/motor plans from the hippocampus and PFC respectively, and its output controls goal-directed behaviors (see Goto and Grace, 2008 and references therein). Behavioral studies in animals revealed that the NAc generates learned or instrumental behaviors elicited by cues predicting natural or drug reward (Everitt and Wolf, 2002; Kelley and Berridge, 2002). The GABAergic NAc projections to the ventral pallidum also modulate or control adaptive motor behaviors (Zahm, 2000). Adaptive motor responses are generated as a result of an organism, having obtained information from its external environment and internal cues, interpreting and utilizing both factors in producing an appropriate behavior supporting the survival in changing circumstances. For example, obtaining food in nature by wild animals in absence or presence of their predators, or lever pressing by laboratory animals in a particular pattern that would result in to a food or drug reward. NAc is further divided into functionally and anatomically distinct sub-regions, the NAc core (AcbC) and shell. Pharmacological inactivation of the AcbC reduces the response to rewarding cues in rats, suggesting its involvement in regulating goal-directed behavior, while the shell facilitates alterations in behavior (task switching) in response to changes in the incentive value of conditioned stimuli, suggesting an overall adaptive function of NAc for obtaining rewards (see

Ambroggi et al., 2011 and references therein). Up to this point we have seen that NAc is the key regulator of reward-related behaviors and these behaviors are generated by changes in neuronal activity in the NAc. This neuronal activity of MSNs in the NAc, as mentioned above, is directly dependent on the upstream regions like the PFC, sending direct glutamatergic inputs in to it.

1.6.2 Prefrontal cortex

The PFC consists of glutamatergic pyramidal neurons representing around 80% of the neuronal population while 20% are GABAergic interneurons (Zhang, 2004; Chang and Luebke, 2007). The cortical pyramidal neuron population is diverse based on their size, dendritic morphology, and firing properties. These neurons are grouped into two classes: 1) regular spiking neurons that show little or slower adaptation during repetitive firing and 2) intrinsically bursting neurons that display more complex patterns of firing. In the prefrontal cortex of both rodents and primates, the regular spiking neurons are further divided in three types: regular spiking 1 and 2 and fast accommodating neurons based on their responses to current injections. Regular spiking 1 respond to current injection by smooth increases in action potential frequency with little change in action potential threshold or amplitude. Regular spiking 2 neurons show decreasing action-potential height and an increase in action potential firing threshold whereas Fast accommodating neurons show similar changes in action potential characteristics along with a robust accommodation in spiking. Intrinsically bursting neurons make up only a small percentage of neocortical pyramidal neurons and generate three to six spikes during threshold amounts of stimulation.

GABA interneurons are divided in four different classes based on spiking patterns during current injection (Kawaguchi, 1993b and 1995): a) fast-spiking neurons are in majority and display rapid action potentials (~0.5 ms half-width) each followed by a deep after-hyperpolarization, b) late spiking interneurons display a slowly developing depolarization during current injection that eventually triggers spikes, c) low threshold spike neurons generate action potentials upon current injection only when cells are held at fairly hyperpolarized potentials (<–70mV) and d) regular spiking non-pyramidal neurons show a slow adaptation to firing and have prominent hyperpolarization-induced inward currents.

The Rat PFC can be subdivided in to medial, later and orbital structures. The medial PFC (mPFC) is further divided into four parts: the medial agranular, anterior cingulate, prelimbic and infralimbic cortex (Berendse and Groenwegen, 1991; Hoover and Vertes, 2007). PFC organizes behavior in a sequential manner and it is involved in executive function involving attention, planning, and decision making. The rodent PFC has been a subject of dispute and the main issue of controversy is whether rats have a prefrontal area that is comparable with the PFC of primates. In their review, Uylings and colleagues (2003) established that rodents do in fact have a region of the frontal cortex which could be defined both anatomically and functionally as PFC and is comparable to the dorsolateral PFC of primates and humans (also see Ongur and Price, 2000 and references therein). Furthermore, another review by Seamans and colleagues (2008),

describing electrophysiological findings, have taken this a step further by suggesting that the rat medial PFC combines rudiments of the both primate dorsolateral PFC and anterior cingulate cortex at an elementary level which in primates may perhaps have formed the building blocks required for evolutionary expansion of the PFC dorsally. Recent reviews have projected different regions of PFC modulating the fear and reward related behaviors. (see Peters et al., 2009; Richard and Berridge, 2013 and references therein).

1.7 Dopaminergic neurotransmission and drugs of abuse

The dopaminergic neurons originating in the ventral tegmental area (VTA), project to the NAc, PFC, basolateral amygdala (BLA) and extended amygdala (Dahlström and Fuxe, 1964; see Koob, 1992 and references therein). The mesolimbic dopamine system (VTA to NAc) has been shown to be activated in both rodents and primates by various natural rewards like food, water, sexual activity and during expectation of these events as well (Wise and Leeb, 1993; Schultz et al., 1997). In addition, all drugs of abuse including alcohol (Imperato and Di Chiara, 1986; see Di Chiara and Imperato, 1988; Hemby et al., 1995) increase dopamine in the NAc through various mechanisms (see Table 1). However, it should be noted that lesions of the mesolimbic dopamine neurons do not completely abolish alcohol-reinforced behaviors in animals implying that dopamine might be necessary for alcohol reinforcement but not essential in mediating alcohol reinforced behaviors (Rassnick et al., 1993).

Thus, although dopamine is important in mediating reinforcements dependence-producing drugs do not only interact with the dopaminergic system but also affect other neurotransmitter systems directly or indirectly including the excitatory glutamatergic system.

Drugs of abuse	**Mechanism of dopamine release in NAc**
Stimulants (Cocaine, amphetamines)	Block dopamine transporters (DAT) or increase dopamine release from terminals
Opiods (heroin)	Disinhibits inhibition of dopaminergic neurons in VTA via μ-opioid receptors.
Nicotine	Direct and indirect activation of VTA dopaminergic neurons through pre- and postsynaptic nicotinic receptors on glutamatergic terminals and on VTA dopaminergic neurons.
Alcohol	Multiple targets, facilitates GABA inhibitory neurotransmission, disinhibits VTA dopaminergic neurons from GABA interneurons or affects the glutamate terminals in the VTA, which are directly regulating dopamine release in the NAc.

Table 1. How different drugs may increase dopamine release in NAc.

1.8 Effects of ethanol on glutamatergic transmission

The NAc receives glutamatergic inputs from various brain regions like PFC, basolateral amygdala (BLA), hippocampus and hypothalamus (see Sesack and Grace, 2010 and references therein) mediating goal-directed behavior. Abnormal plasticity in these glutamatergic pathways has been suggested to underlie the course of addiction (see Kalivas and O´Brien, 2008 and references therein). Supporting the involvement of glutamatergic transmission in addiction, studies done by Pierce and colleagues (1996) showed that repeated cocaine injections increased glutamate release in NAc and produced locomotor sensititzation. A microinjection of the AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in the NAc reduced this motor activity only in cocaine-sensitized rats but not in control and non-sensitized rats (Peirce et al., 1996). The increase in motor activity with different drugs have been directly associated with the potency of that particular drug in establishing addiction. Following the above mentioned study, the role of AMPA receptors in expression of cocaine-induced behavioral sensitization was further consolidated as it was shown that AMPA receptor blockade, in the AcbC, prevents reinstatement to cocaine or cue-prime (Cornish and Kalivas, 2000; Di Ciano and Everitt, 2001). Along with sensitization studies, the reinstatement of drug-seeking, which is defined as resumption of a previously drug-reinforced behavior by a priming exposure to drug or non-drug stimulus after extinction, has also been associated with increased glutamate release in the NAc (McFarland et al., 2003).

Ethanol is known to inhibit ionotropic glutamate receptor activity in the brain and NMDA receptors are the major target of ethanol's inhibitory action (Lovinger et al., 1989, see Lovinger and Roberto, 2013). AMPA and KA receptors are blocked as well, although, higher concentrations of alcohol are needed as these receptors are less sensitive to alcohol than NMDA receptors (Lovinger, 1993; Nie et al., 1994; Valenzuela et al., 1998). Ethanol, which is also known as a non-competitive inhibitor of NMDA receptors (Morrisett et al. 1991), also interacts with various factors modulating NMDA receptors' activity, for example extracellular Mg^{2+} and glycine (Rabe and Tabakoff, 1990; Morrisett et al., 1991), intracellular Ca^{2+}(Dildy-Mayfield and Leslie, 1991; Mirshahi et al., 1998) and phosphorylation of NMDA receptors by protein kinases like Fyn (Miyakawa et al., 1997; Yaka et al., 2003), PKA (Ferrani-Kille et al., 2003), PKC (Snell et al., 1994) and DARPP-32 (Maldve et al., 2002). As a result of inhibitory effects on ionotropic receptors, especially the NMDA receptors, ethanol also inhibits the induction of neural plasticity in many brain regions including the dorsal (Yin et al., 2007) and ventral striatum (NAc) (Nie et al., 1994). Microdialysis studies in rats showed that acute alcohol administration induces a biphasic effect on glutamate release in the NAc with an initial inhibition of glutamate release followed by a delayed increase (Moghaddam and Bolinao, 1994; Kashkin and De Witte, 2004). Along with the postsynaptic targets, acute alcohol has been suggested to inhibit glutamatergic transmission in adolescent and pre-adolescent animals via decreased glutamate release (Nie et al., 1994; Steffensen et al., 2000; Hendricson et al., 2003; Roberto et al., 2004).

In the present study I have looked at effects of ethanol on glutamatergic transmission in the NAc and the PFC. The PFC functions have been suggested to be severely disturbed in alcohol addicts and associated with the loss of control on alcohol intake (craving and relapse) (see Kalivas and Volkow, 2011) . Despite its important role, there have been surprisingly few studies investigating the effects of ethanol in this region of the brain. Electrophysiological studies performed by Tu and colleagues (2007) in slice co-cultures showed that the regular intrinsic firing activity in PFC was NMDA receptor-dependent and was blocked after ethanol treatment. Interestingly, they also showed that during the washout period after ethanol treatment the firing activity was potentiated in the slice co-cultures (Tu et al., 2007). In follow-up, *in vivo* experiments done in pre-adolescent animals the authors concluded that ethanol-mediated changes in the firing activity was regulated only post-synaptically and the authors did not observe any presynaptic modulation of glutamate release (Weitlauf and Woodward. 2008). As the PFC receives glutamatergic inputs from different brain regions like hippocampus, BLA and hypothalamus, and acute alcohol has been shown to inhibit the glutamatergic transmission in both the hippocampus (Lovinger et al., 1989) and the BLA (Roberto et al., 2004), it could be expected that ethanol treatment in an intact animal would affect (decrease) the glutamate release in the PFC. However, a microdialysis study performed by Selim and Bradberry, (1996) did not observe any changes in the PFC glutamate levels after acute alcohol injection in rats. We have studied the ethanol's effect on glutamate neurotransmission based on age (adolescent versus adults) looking at the glutamatergic neurotransmission in the NAc using field recordings and glutamate release in the PFC using micoelectrode array amperometry.

2. Aims of the study

1) To study the glutamatergic transmission in adolescent and adult NAc and their modulation by ethanol using extracellular electrophysiological recordings in brain slices.

2) To examine the effects and targets of acute-ethanol, modulating the glutamatergic transmission (LTP) in the NAc brain slices using extracellular electrophysiology.

3) To investigate the age related differences in PFC glutamate dynamics and its modulation by acute systemic ethanol injection.

3. Materials and Methods

3.1. *In vitro* slice experiments

3.1.1. Animals

Male C57BL/6 (Charles River, Germany) mice aged 22–30 days (adolescents) and 5–8 months (adults) (Laviola et al 2003) were used in the experiments. The experiments were approved by Stockholms norra djurförsöksetiska nämnd, ethical permit number: N176/08 and N268/08. Animals were maintained in a temperature and humidity controlled facility on a 12:12-h light/dark cycle (lights on at 6 AM) and had free access to food and water. Adolescent animals were 21 days when they arrived and were used within 9 days after arrival.

3.1.2. Materials

Ethanol (95%, Solveco AB, Stockholm, Sweden) was diluted in artificial cerebrospinal fluid (aCSF) to a final concentration of 20, 50 or 100 mM. All chemicals for preparing the aCSF-solution, sodium chloride (NaCl), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), glucose ($C_2H_{12}O_6$) and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma (Stockholm, Sweden). Drugs used in the experiments: NMDA (NMDA receptor agonist), (S)-3,5-dihydroxyphenylglycine (DHPG, mGluR group I agonist), CGP 55845 ($GABA_B$ receptor antagonist), bicuculline ($GABA_A$ receptor antagonist), muscimol ($GABA_A$ receptor agonist), SKF 97541 ($GABA_B$ receptor agonist) were purchased from Tocris Bioscience (Bristol, UK).

3.1.3. Preparation of brain slices

Animals were anesthetized with isoflurane followed by cervical dislocation and decapitation under anaesthesia. The brains were rapidly removed and coronal brain slices containing the NAc, striatum and the overlying cortex (400 µm thick, figure 1a) were cut using a micro slicer (VT 1000S, Leica Microsystem, Heppenheim, Germany). Slices were incubated for at least 1 hour, at 32°C in oxygenated (95% O_2 + 5% CO_2) artificial cerebrospinal fluid (aCSF containing 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$,10 mM glucose and 26 mM $NaHCO_3$, pH 7.4). Slices were then transferred to a recording chamber (Warner Instruments, Hamden, CT) and continuously perfused with oxygenated aCSF at 28°C. The recording chamber was mounted on an upright microscope (Olympus, Solna, Sweden).

3.1.4. Extracellular field potential recordings

Extracellular field recordings or field excitatory postsynaptic potentials/population spikes (fEPSP/PS) were recorded using a borosilicate glass micropipette (low resistance; G120F-3; Warner Instruments) filled with aCSF positioned on the slice surface. A concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) placed near the recording electrode were used for evoking synaptic responses (fEPSP/PS) in the brain slice.

To evoke fEPSP/PS, a single electrical stimulus, 0.1 ms in duration, was applied every 15 seconds at an intensity of 40-60% of the maximal response as established by an input/output curve from each individual slice before beginning of the recording session (figure 1b). A stable baseline was recorded for 20-30 minutes (slices were discarded if stable baseline could not be achieved within an hour) before applying drugs or high frequency stimulation (HFS).

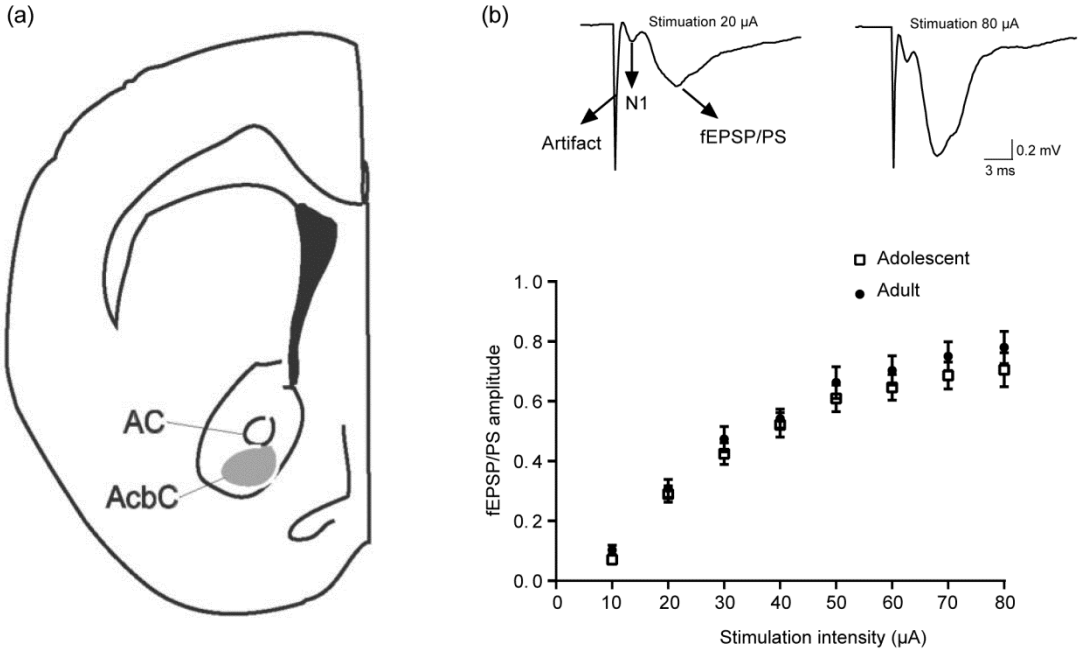

Figure 1. Coronal brain section showing the NAc region where recordings were performed and input-output curve was plotted for each slice before starting the baseline recordings. a) The grey region showing the nucleus accumbens core (AcbC) where all the recordings were performed, AC is the anterior commissure. b) The amplitude response of fEPSP/PS increased with increasing stimulation current (µA). 40-60% of the maximal response stimulation intensity was used during the experiments. Also showing are the typical fEPSP/PS traces, with a negative N1 and fEPSP/PS response. CNQX (AMPA/kainite antagonist) has been shown to block the fEPSP/PS component which showed that this was an AMPA/kainate receptor mediated response (Schotanus et al., 2006). Also, the N1 was unaffected by CNQX demonstrating that it is a non-synaptic and glutamate release independent component. Also, the time-interval between the stimulation artifact and fEPSP/PS response, suggests the fEPSP/PS to be a monosynaptic event.

Signals were acquired at 10 kHz, filtered at 2 kHz and amplified 500 times via an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), and recorded on a Dell computer (Stockholm, Sweden) using acquisition and data analysis software from Axon Instruments (pClamp9). Drugs were applied in known concentrations in the perfusion solution by switching a three-way tap. The perfusion flow rate was 1-2 ml/min

3.2. *In vivo* experiments measuring glutamate release in freely moving animals

3.2.1. Animals

Male Sprague Dawley rats (Charles River, Germany) postnatal day: 30 to 55 and adults: 3-4 months old were used in our experiments. The experiments were approved by Stockholms norra djurförsöksetiska nämnd, ethical permit number: N319/12, N457/12 and N602/12. Animals were maintained in a temperature and humidity controlled facility on a 12:12-h light/dark cycle (lights on at 6 AM) and had free access to food and water. Animals were acclimatized to the environment for 3-5 days before being used in any experiments. All the experiments were performed during day time between 6 AM and 6 PM.

3.2.2. Materials

L-ascorbic acid (AA), dopamine, L-glutamate (monosodium salt), glutaraldehyde [25% (w/v) in water], bovine serum albumin (BSA), *m*-phenylenediaminedihydrochloride (*m*- PD) and hydrogen peroxide (H_2O_2) were ordered from Sigma Aldrich Corp. (St. Louis, MO, USA). Sodium chloride (NaCl) was purchased from VWR Leuven, Belgium. L-Glutamate oxidase (GluOx; EC 1.4.3.11) was purchased from Heamochrom Diagnostics AB, Gothenburg, ethanol (95%) was purchased from Solveco AB, pentobarbitalnatrium was bought from Apoteket AB and isoflurane was purchased from Baxter Medical AB, all companies based in Sweden. Ethanol was dissolved in physiological saline for intraperitoneal injections (i.p.). Other solutions were prepared using distilled and deionized water.

3.2.3. Microelectrode structure and enzyme coating for glutamate specificity

The microelectrodes consists of a ceramic paddle with four platinum recording sites and polyimide insulation that are suitable for measurement of H_2O_2 (for details about structure and assembly of the microelectrodes see Konradsson-Geuken et al., 2009) The sites are arranged in two pairs beginning approximately 100 µm from the electrode tip. To design a glutamate sensitive microelectrode, one pair of recording channel sites was coated with a mixture of GluOx (2%, 0.5 unit/1 µL), BSA (1%) and glutaraldehyde (0.125%) mixture and the remaining pair was coated only with BSA (1%) and glutaraldehyde (0.125%) and they served as control sentinel channels sensitive to the oxidation of endogenous molecules other than glutamate (figure 2). Enzyme coated microelectrodes were allowed to dry for 48 hours at room temperature.

3.2.4. *m-PD* plating of microelectrodes

Prior to calibration *m*-PD (5.0 mM) was electropolymerized onto all sites of the microelectrode in order to reduce access of potential electroactive interferents, like AA and catecholamines, to the

platinum recording sites (Mitchell, 2004). The electroplating was done in nitrogen bubbled phosphate-buffered saline 0.05 M, using the fast analytical sensing technology (FAST-16) electroplating tool (peak-to-peak amplitude of 0.25 V every 0.05 s for 22 minutes).

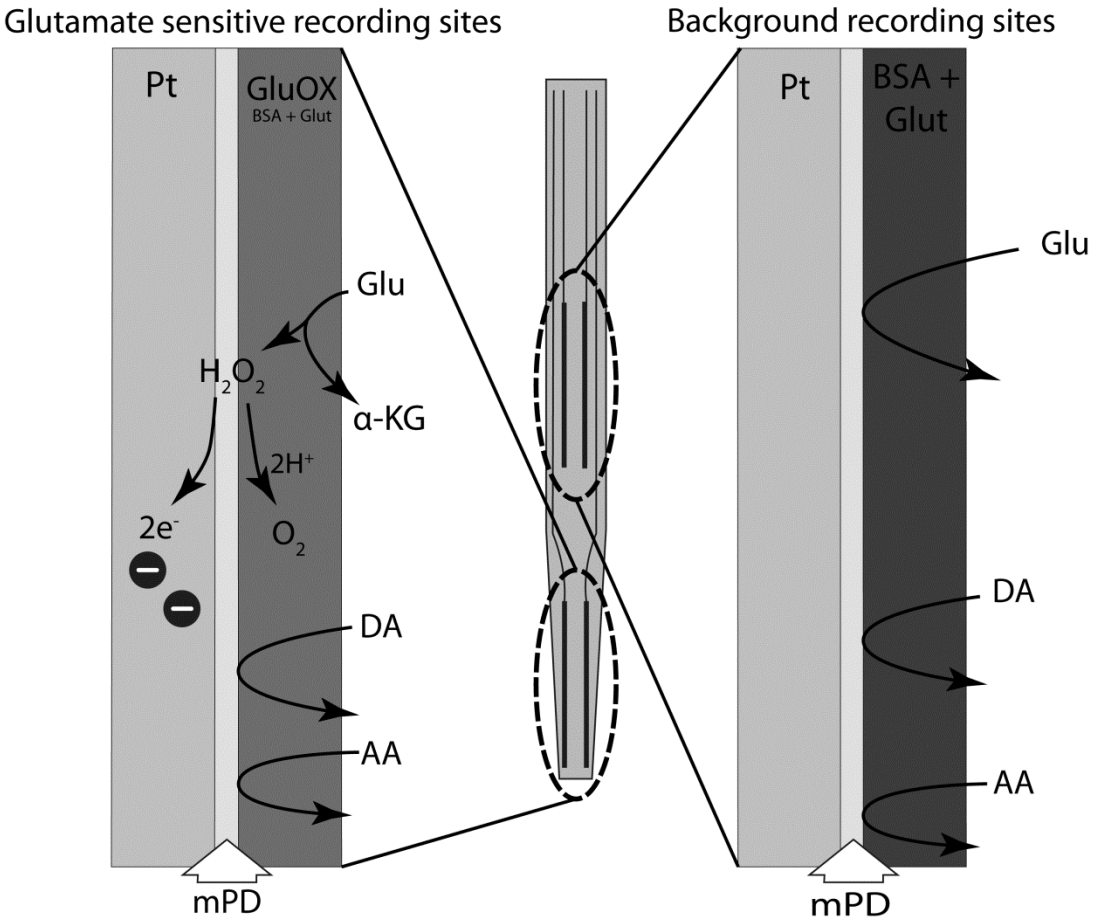

Figure 2. A schematic picture showing the enzyme coating of a glutamate sensitive microelectrode.

The *m*-PD plating and the selective enzyme coating of the microelectrode allows for a self-referenced recording in which the current derived exclusively from glutamate oxidation can be isolated (see Day et al., 2006; Rutherford et al., 2007; Konradsson-Geuken et al., 2009; 2010). The released glutamate gets oxidized by GluOx at the glutamate-sensitive sites, generating α-ketoglutarate and H_2O_2. Since the microelectrode is maintained at a constant potential (+0.7 V versus an Ag/AgCl reference), the H_2O_2 reporting molecule is further oxidized, yielding two electrons. The resulting current is then amplified and recorded by a FAST-16 recording system (Quanteon, LLC, Nicholasville, KY, USA). Extracellular glutamate reaches the platinum surface of control sentinels (without GluOx) but no oxidation current is generated. Therefore, any current detected at these sites is due to electrochemically active interferents other than glutamate.

3.2.5. *In vitro* calibration of microelectrodes

Microelectrodes were calibrated *in vitro* immediately prior to implantation and representative trace is provided below (figure 3). Calibrations were performed in a stirred solution of phosphate buffered saline (0.05 M, 40 mL, pH 7.4, 37 °C). A stable baseline was established, AA (250 µM), three aliquots of glutamate (20mM; resulting in a final concentration of 20, 40 and 60 µM), DA (2 µM), and H_2O_2 (8.8 µM) were sequentially added to the calibration beaker. Amperometric signals were acquired at a rate of 2.0 Hz. The sensitivity (pA/µM glutamate), limit of detection (LOD) for µM glutamate concentration (i.e. the smallest signal in glutamate concentration detected), selectivity (ratio of glutamate over AA), and linearity (R^2) were calculated. The microelectrodes to be used for implantation and further *in vivo* recordings had to fulfil the following calibration criteria: (i) similar background current (i.e. less than 20 pA difference between the glutamate-sensitive and control sentinel channels), (ii) linear response to increasing concentrations of glutamate (R^2 close to 1), (iii) a minimum glutamate sensitivity of -0.003 nA/µM glutamate, (iv) a limit of detection of ≤0.5 µM, and (v) a high selectivity for glutamate over AA and dopamine (i.e. >50:1).

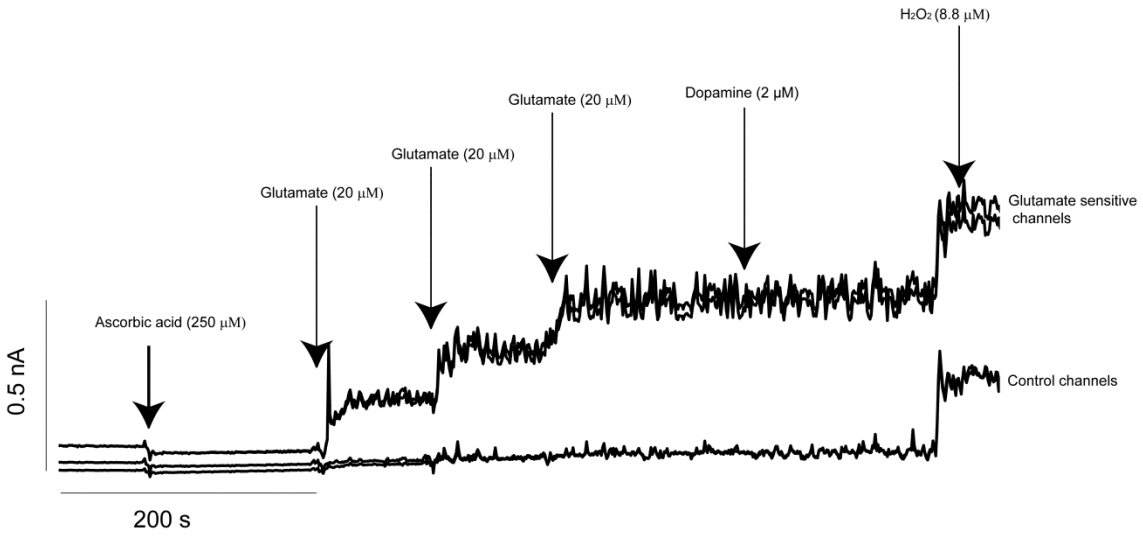

Figure 3. A representative *in vitro* calibration of the microelectrode performed immediately prior to implantation into the PFC. The top two traces are the glutamate-sensitive channels (GluOx coated) and the bottom two traces are generated from the control channels. Arrows represent the addition of various substances into the calibration beaker. Current (nAmperes) is shown along the vertical bar and time (seconds) by the horizontal bar. Three successive additions of glutamate (20 µM/aliquot) produced a linear increase of current on glutamate sensitive channels. Expectedly, there were no changes detected on the two control channels. The calibration also shows comparable sensitivities on all four channels to the reporting molecule H_2O_2. *m*-PD was efficient in blocking the changes in currents due to potential electroactive interferents, i.e. AA and dopamine.

3.2.6. Surgery and implantation of microelectrode

Animals were handled for 5 days and habituated to saline injections (6 ml/kg/day) for 3 days. Surgery was performed under continuous anaesthesia (isoflurane with air (30% O_2 and 70% N_2) 1-3 L/min, 2-4% v/v). A microelectrode was unilaterally implanted in the PFC in adolescent animals postnatal day: 30-35 at anterior-posterior (AP)+3.0 from bregma, medial-lateral

(ML)±0.8 mm from midline, and dorsal-ventral (DV)-5.3 mm from dura; PND 45-55 at AP+3.1 mm from bregma, ML±0.9 mm from midline, and DV-5.8 mm from dura (Boyce and Finlay, 2009) respectively and in adult animals AP+2.7 mm from bregma, ML: ±0.6 mm from midline, DV -3.9 mm from dura (Konradsson-Geuken et al., 2009; Konradsson-Geuken et al., 2010). The stereotaxic coordinates for adults were from Paxinos and Watson brain atlas, fourth edition, 1998. An Ag/AgCl reference electrode was implanted in a contralateral site from the recording area (figure 4). The microelectrodes were fixed using dental cement and stainless steel screws. Animals were allowed to recover for 48 hours after surgery (figure 4).

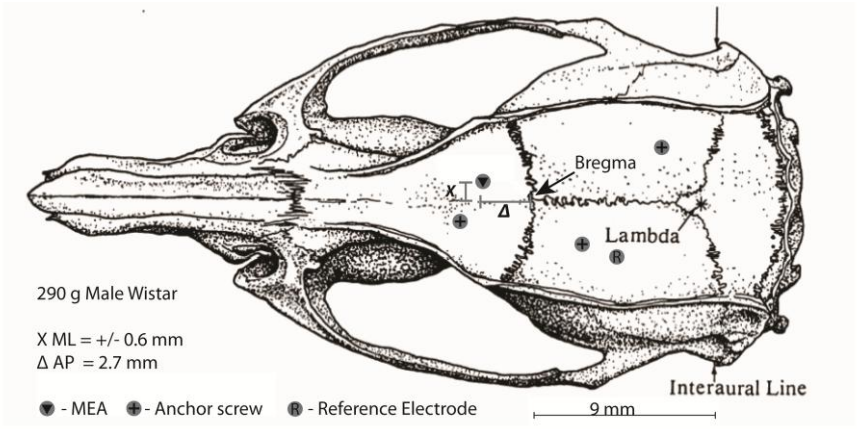

Figure 4. A schematic picture of an adult rat skull showing coordinates and reference points (bregma) for surgical implantation of microelectrode, reference electrode and placement of anchor screws. The dimensions are provided for the location of PFC from bregma where the microelectrodes were implanted. ML : medial-lateral; AP : anterior-posterior. Modified from Paxinos and Watson, fourth edition, 1998.

3.2.7. *In vivo* recordings with acute intra-peritoneal injection

Recordings were conducted during daytime in freely moving rats in a wooden box (55 cm H 51 cm W 55 cm L). Animals were placed in the recording box and connected to the head stage. On the first day (experimental day 1) of recording, stable baseline signals were recorded for 3–4 hours followed by an i.p. injection of physiological saline (6 ml/kg) and recording was continued for an additional three hours. The same procedure and time schedule was repeated on the second day (experimental day 2) with an i.p. injection of ethanol (1 g/kg).

3.2.8 Histological verification of microelectrode placement

Brains were removed and stored in 25% sucrose 4% formaldehyde solution at 4°C for at least 24 hours. Coronal brain sections of 50 µm were cut using a cryostat and mounted on gelatin-coated slides, stained using neutral red and examined under a light microscope for verification of microelectrode placement.

3.2.9. Data analysis using FAST analysis

The glutamate signal, initially measured in pA, was converted to a concentration equivalent (μM) with FAST analysis software version 4.4, on the basis of the individual calibration curves generated immediately before surgery. The basal levels were calculated based on the difference between glutamate sensitive and control sentinel as previously described (Burmeister and Gerhardt, 2001; Day et al., 2006; Rutherford et al., 2007). Glutamate levels were reported as an average of 15 minutes of recordings during baseline before injection and after injection from each first, second and third hour. Glutamate transients, i.e. spontaneous glutamate peaks with a total time interval of 1-3 seconds, were sorted using control R^2 value which is a metric correlation between the glutamate and control channel. A high R^2 value (close to 1) meant that glutamate and control sites are closely correlated and the signal is not electrochemical i.e. the signal observed is not due to glutamate release, rather electrical noise from the surroundings. Only the peaks with R^2 values ranging from 0.1 – 0.55 were selected for further analysis. Additionally, the peak threshold value was set to 3 times the baseline noise values for selecting glutamate transients. All experiments were recorded at 2 Hz.

3.3. Statistical analysis

3.3.1 *In vitro* experiments

The p-clamp 9 system averaged four responses (following stimulations) as one data point for every minute. Numerical values were expressed as means ± standard error of the mean (SEM) for *in vitro* slice experiments and effects of alcohol and various drugs was expressed as mean percentage change from the average of 20 minute baseline amplitude (100%). Data were evaluated for significance using the two-tailed Student's t-test for paired and unpaired observations and two-way analysis of variance (ANOVA) followed by the Bonferroni post-test was perfrmed when required. All the statistical tests were performed using GraphPad Prism 6.0 software.

3.3.2 *In vivo* experiments

Data was evaluated for significance using the two-tailed Student's t-test for paired and unpaired observations using GraphPad Prism version 6.01.

4. Results and Discussion

4.1. Acute ethanol mediated depression of glutamatergic transmission is stronger in adolescents compared to adult brain slices (Paper I).

The neurons in NAc (medium spiny neurons) are not uniformly oriented as in the hippocampus; therefore, we used and compared fEPSP/PS amplitudes rather than slope (Misgeld et al., 1979) for evaluating the effect of various drug treatments in the NAc. It was previously demonstrated that the fEPSP/PS responses are blocked by application of CNQX in the bath (Schotanus et al. 2006) and therefore respresents monosynaptic glutamatergic neurotransmission. The fEPSP/PS responses were evoked using electrical stimulations in brain slices containing AcbC region being continuously perfused with aCSF. The amplitude of the fEPSP/PS was dependent on stimulation intensity but there was no difference in amplitudes between slices taken from adolescent and adult mice.

Ethanol (50 mM) dissolved in aCSF was perfused for 20 minutes and was consistently found to induce a maximum depression of fEPSP/PS amplitudes (figure 5). In adolescent brain slices, ethanol concentrations of 20 mM had no effect while 100 mM ethanol induced a lower depression in fEPSP/PS compared to the depression induced by 50 mM. Moreover, the depressant effect of 50 mM ethanol was more prominent in brain slices from adolescents compared to that observed in brain slices from adult mice (figure 5a and 5c). In adolescent mice, ethanol (50 mM) decreased the amplitude of the fEPSP/PS to 78.6 ± 1.7% (n=23 slices), whereas, in adults this the amplitude decreased to 92.8 ± 3.2% from the baseline (n=10). In both age groups the depressant effect of ethanol was statistically significant and the depression in adolescent fEPSP/PS mediated by 50 mM ethanol was significantly stronger than that observed in adults (figure 5d).

Additionally, using a paired-pulse protocol (two single stimulation pulses applied with a 20-ms interval) in adolescent brain slices we observed that the ratio between the second fEPSP/PS and the first fEPSP/PS was significantly increased to 117.4 ± 3.4% of baseline ($P < 0.001$, figure 5b) indicating presynaptic modulation of glutamate release.

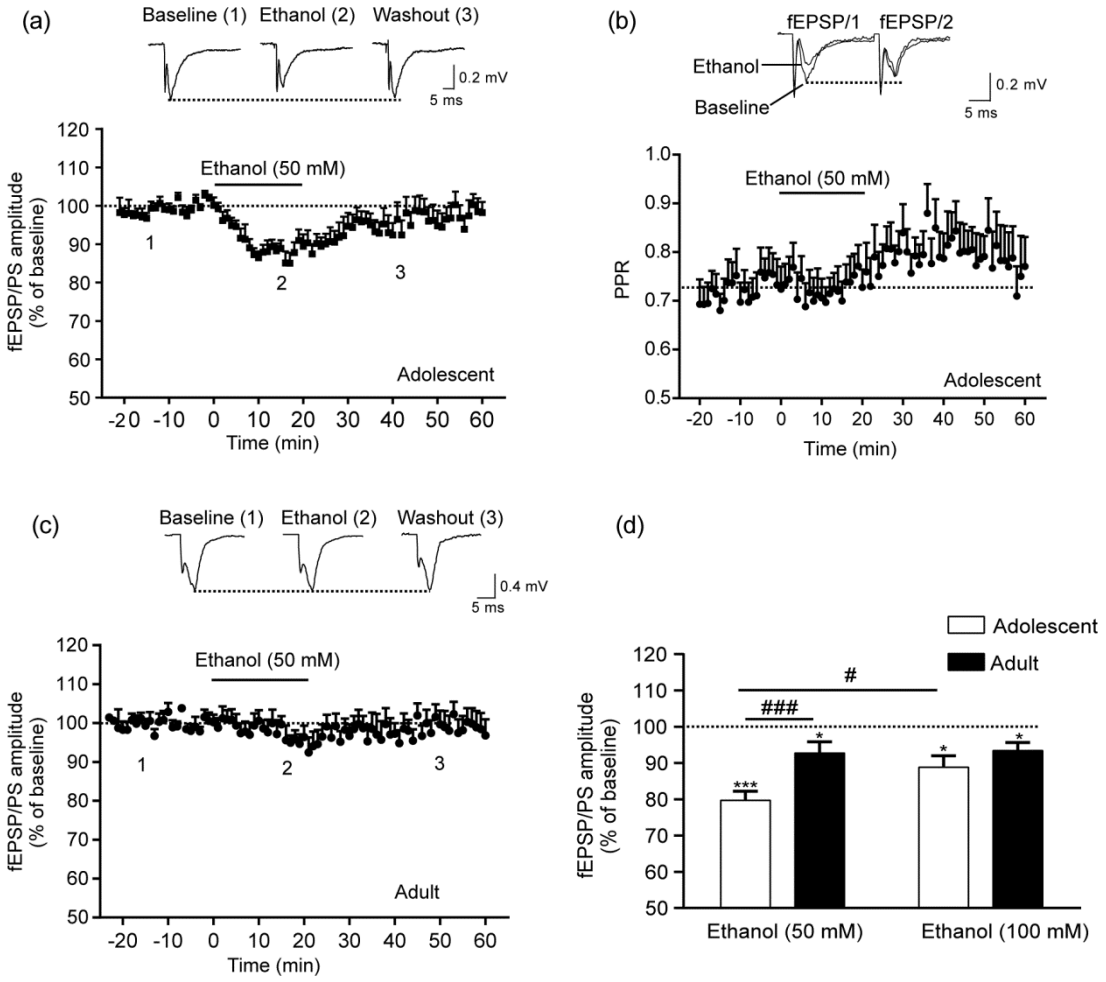

Figure 5. Ethanol produces a stronger inhibition of glutamatergic synaptic transmission in the nucleus accumbens (NAc) of adolescent mice as compared with adult mice. (a) Ethanol (50 mM), applied in the perfusion solution at the time indicated by the horizontal bars, decresed amplitude of the fEPSP/PS (mean ± SEM) in adolescent (a) and adult (c) mice. (b) The paired-pulse ratio in adolescent mice was increased during ethanol (50 mM). Representative traces of fEPSP/PS are showed above respectively for figure (a), (b) and (c). (d) Bar graph illustrates the maximal depressant effect of ethanol (50 mM and 100 mM) measured in individual slices in adolescent and in adult mice. **P* < 0.05, ****P* < 0.001 (paired *t*-test), #*P* < 0.05, ###*P* < 0.001 (analysis of variance).

These results showed that ethanol at a 50 mM concentration affects glutamatergic transmission in adolescent animals more prominently than in adults. Using the paired-pulse ratio we also confirmed previous findings that ethanol depresses glutamatergic synaptic transmission in the NAc through a presynaptic mode of action (Nie et al., 1993; Siggins et al., 2005). The nucleus accumbens receives glutamatergic inputs from several brain regions including the PFC, BLA amygdala, ventral hippocampus and thalamus. While neither projection is targeting either the core or shell subregion exclusively, anatomical and functional data indicate that glutamatergic inputs from the prelimbic part of the prefrontal cortex are particularly important in the nucleus accumbens core. Interestingly, these projections have been suggested to mediate drug-seeking behavior (Peters et al. 2009). Provided the important role of altered synaptic plasticity in the nucleus accumbens in addiction (see Gipson et al. 2013a), the higher sensitivity to

modifications in glutamatergic neurotransmission induced by ethanol in adolescent mice may have an impact on synaptic plasticity in this region.

The inhibitory effect of ethanol on glutamatergic transmission in adolescent mice was blocked in the presence of either a $GABA_A$ or a $GABA_B$ receptor antagonist (figure 6). This finding indicates that the effect of alcohol is mediated via release of GABA or a direct activation or potentiation of both $GABA_A$ and $GABA_B$ receptors. Indeed, previous studies have consistently demonstrated that ethanol may potentiate GABAergic transmission, partly via increased release of GABA and partly via direct interaction with GABA receptors (see Weiner and Valenzuela 2006).

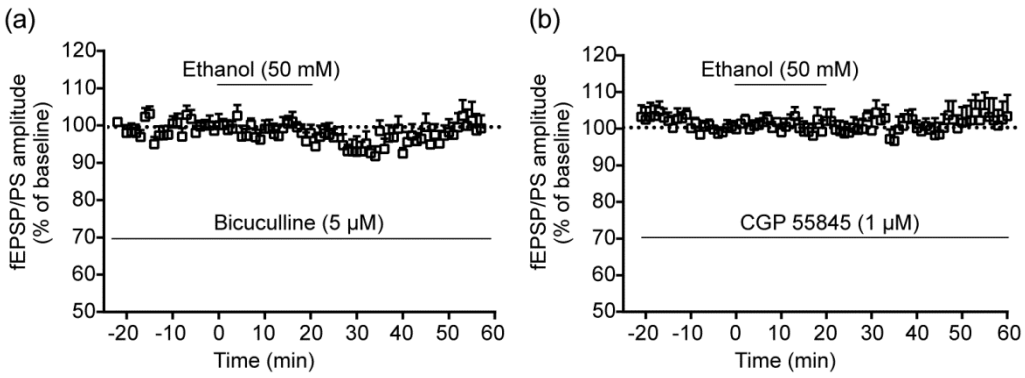

Figure 6. GABA receptor antagonists block ethanol mediated inhibition. a) $GABA_A$ receptor antagonist bicuculline (5 µM) and b) $GABA_B$ receptor antagonist CGP 55845 (1 µM) applied throughout the fEPSP/PS recording blocked the ethanol (50 mM, applied for 20 mins) mediated inhibition of fEPSP/PS.

4.2. Acute ethanol blocks induction of LTP in NAc core of young animals (Paper II)

As discussed above, we were able to show that ethanol inhibits glutamatergic transmission via presynaptic mechanisms by inhibiting glutamate release. This inhibition was particularly prominent in adolescent mice. HFS has been shown to induce glutamate release (Calabresi et al., 1995) and lead to a long-lasting potentiation of glutamatergic transmission, i,e LTP, which is thought to be an artificial correlate of learning and memory (reviewed by Malenka and Bear, 2004). To test whether ethanol's inhibitory effect on glutamate release in the NAc would also affect synaptic plasticity, we investigated the effect of ethanol on HFS-induced LTP. The HFS consisted of three one-second trains at 100 Hz separated by a 10-second inter-train interval. HFS increased the amplitude of the fEPSP/PS to 126.4 ± 2.3% from baseline and this potentiation was stable for at least 1 hour (n=17). In slices continuously perfused with aCSF containing ethanol (50 mM), HFS did not induce the same degree of LTP. The amplitude of the fEPSP/PS was significantly lower (110.1 ± 3.8%, n=11) when compared to control slices 1 hour after the HFS ($p < 0.001$) (figure 7).

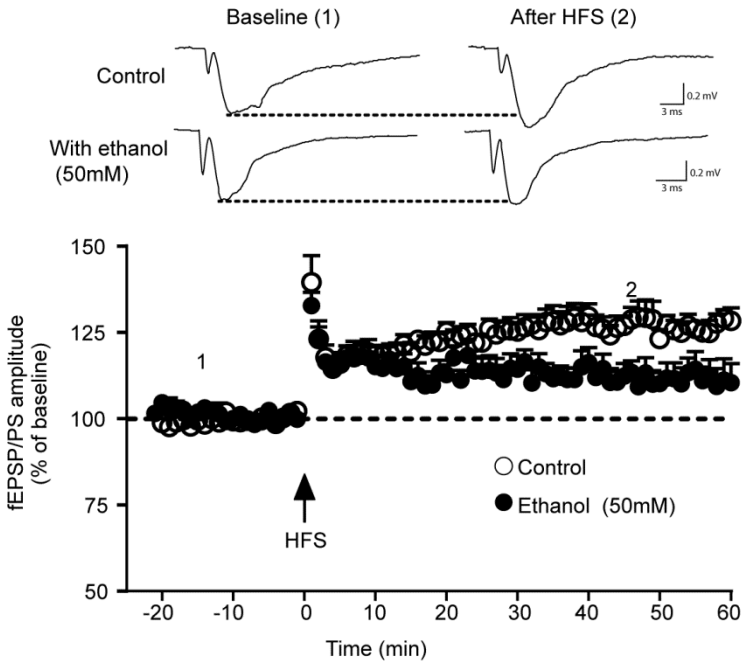

Figure 7. Ethanol inhibits long-term potentiation (LTP) induction in the nucleus accumbens (NAc). High-frequency stimulation (HFS; 3 trains at 100 Hz, 1-second duration, 10-second intervals, applied at the time point indicated by the arrow) induced a long-lasting increase in the amplitude of the fEPSP/PS in control slices, (n=17). HFS did not induce the same degree of LTP in the presence of ethanol (50 mM applied throughout the recording session, n=11). Representative traces of fEPSP/PS recorded in 2 slices (control and perfused with ethanol 50 mM) are shown above the graph at the time points indicated on the graph, before (1) and after (2) HFS.

Additionally, when ethanol was applied after HFS-induced LTP, it depressed the fEPSP/PS amplitude from a potentiated level of 124.7±2.5% to 102.9±2.5% of baseline. The fEPSP/PS amplitude returned to potentiated values (122.9±2.8% of baseline, not significantly different from LTP induced in control slices, $p > 0.05$) following washout of ethanol (figure 8a). The $GABA_B$ receptor antagonist CGP 55845 did not affect the ability of ethanol to inhibit LTP induction. The fEPSP/PS amplitude with CGP 55845 + ethanol (50 mM) (109.8 ± 2.7% of baseline, n=6,) was significantly lower when compared to control experiments with CGP 55845 alone (129.3±7.6% of baseline, n=7 $p < 0.05$, figure 8b).

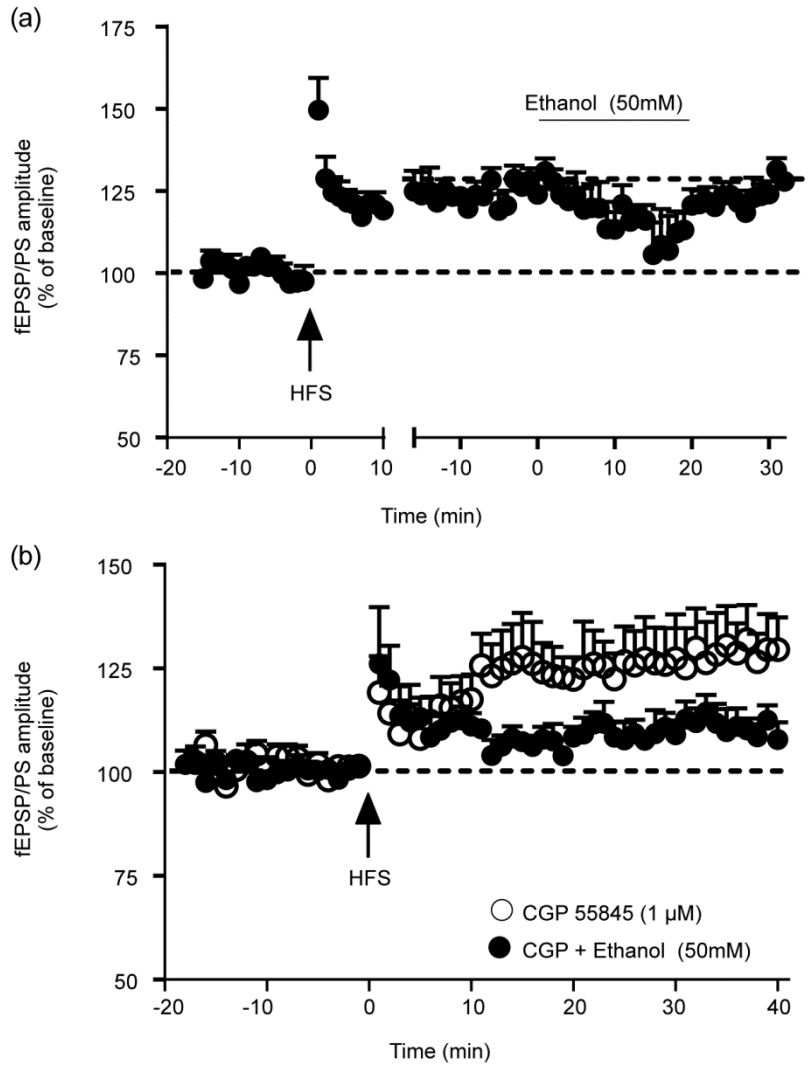

Figure 8. Ethanol-induced inhibition of long-term potentiation (LTP) is not a result of the depressant action of ethanol on glutamatergic neurotransmission. a) Ethanol applied after LTP has been induced depresses the amplitude of the fEPSP/PS but does not affect LTP (n=10). b) LTP is induced in the presence of the GABAB receptor antagonist CGP 55845 (1 µM, n=7). The inhibitory effect of ethanol (50 mM) on LTP induction is not prevented by CGP 55845.

The above results demonstrated that ethanol inhibits induction of LTP. Moreover, the small depressant effect on the fEPSP/PS by ethanol observed in paper I (figure 5a) was persistent even in potentiated synapses however, ethanol did not block pre-induced and stable LTP since the amplitude returned to potentiated magnitudes following washout of ethanol (as shown in figure 7a). Nevertheless, the fact that the $GABA_B$ receptor antagonist did not prevent the ability of ethanol to inhibit induction of LTP suggests that the mechanism by which ethanol blocks LTP is not presynaptic.

4.3. Role of mGluR group I agonist on NMDA-mediated inhibition of glutamatergic neurotransmission (Paper II).

The inhibition of LTP by alcohol did not seem to be due to presynaptic inhibition of glutamate release. Therefore, we sought to investigate whether the mechanism by which ethanol inhibited LTP may be related to the known inhibitory effect of ethanol on NMDA receptors (Lovinger et al., 1989). It has previously been reported that NMDA applied in the perfusion solution during *in vitro* extracellular recordings inhibited glutamatergic synaptic transmission in the dorsal striatum (Schotanus and Chergui, 2008a; Schotanus et al., 2006). Here, we used the same protocol to determine whether activation of NMDA receptors would also depress glutamatergic neurotransmission in the NAc. We found that also in the NAc, NMDA (20 µM, 3 min) depressed the amplitude of the fEPSP/PS to 66.5% ± 7.5% compared to baseline (n=9, figure 9). Surprisingly, in the presence of ethanol (50 mM) NMDA-mediated depression was unaffected. The fEPSP/PS amplitude decreased to 61.5% ± 6.6% (n=10, p>0.05).

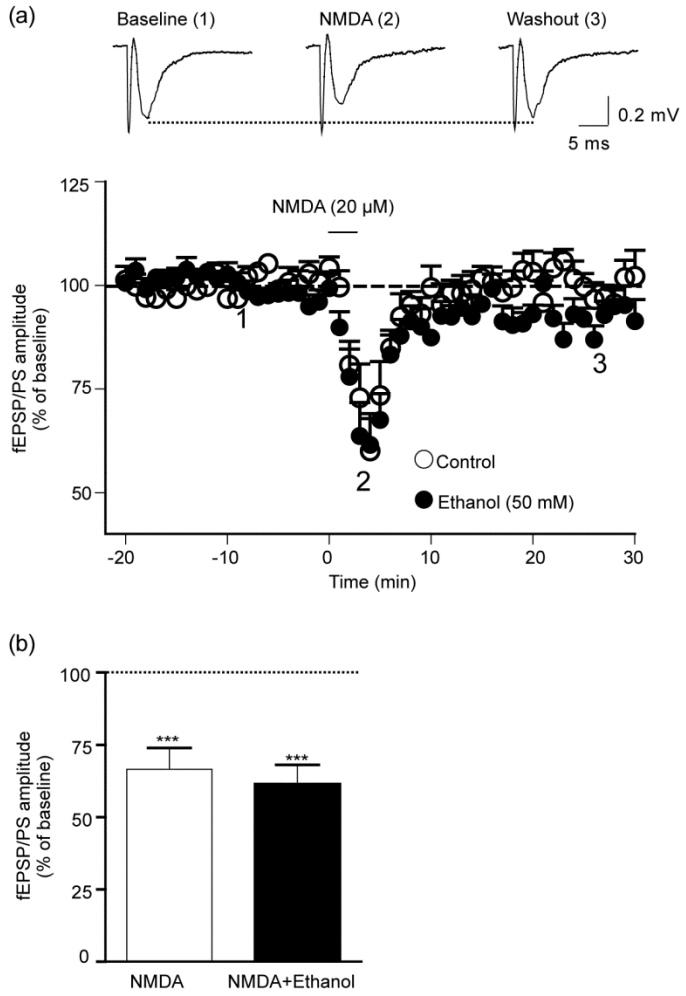

Figure 9. NMDA-induced synaptic depression was unaffected by ethanol. a) NMDA alone (20 µM) bath-applied for 3 minutes significantly depressed fEPSP/PS amplitude (66.5% ± 7.5%, n=9, p<0.05) and in the presence of ethanol (50 mM) throughout the recordings, the NMDA induced depression was also significant (61.5% ± 6.6%, n=13, p<0.05). However, we did not any significant difference between the two effects. b) Bar graph showing the % of depression from the baseline.

As the mGluR group I antagonist DPCPX has been shown to block the induction of LTP in the dorsal striatum (Schotanus and Chergui 2008a and 2008b), and since we did not observe any changes in NMDA-mediated fEPSP/PS depression with ethanol (50 mM), we investigated the role of mGluR group 1 receptors in modulating the NMDA mediated activity. Application of the mGluR group I agonist DHPG (50 µM) throughout the recording sessions, we observed an increased depression to 56.0 ± 7.7% from baseline (n=13) as compared to control slices with NMDA only (74.6 ± 3.1% from baseline, n=11, $p < 0.05$). Our results suggest that mGluR group I receptors potentiate NMDA receptor function and are in line with results of Pisani and colleagues (1997) who showed that NMDA-induced membrane depolarization was facilitated with an mGluR group 1 agonist. Next, we tested the effect of ethanol on this facilitation of NMDA-mediated depression of the fEPSP/PS in the NAc. In the presence of ethanol (50 mM), the potentiating effect of DHPG on NMDA-mediated depression was prevented (80.2 ± 2.6% n=10, $p < 0.05$, figure 10).

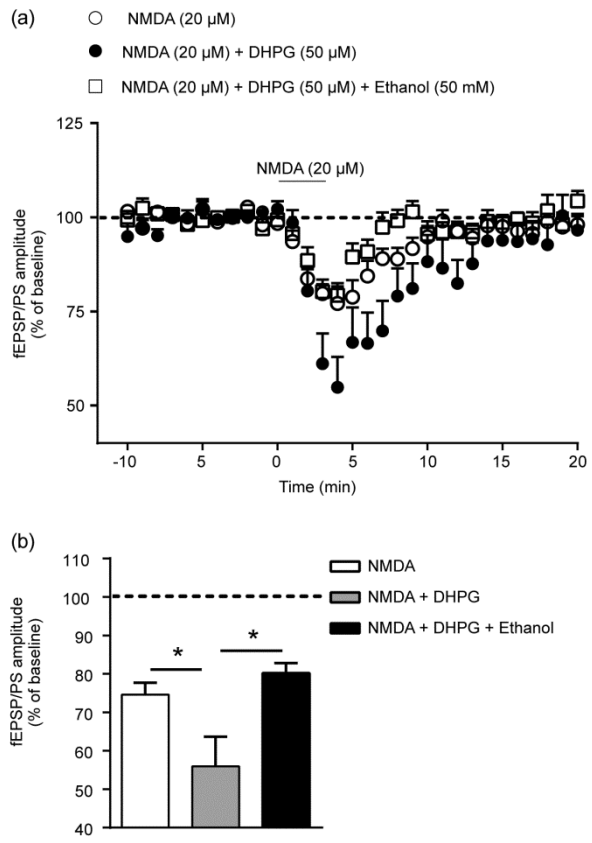

Figure 10. Ethanol blocks the group I mGluR agonist DHPG mediated facilitation of N-methyl-D-aspartate (NMDA)-induced synaptic depression. a) Group I mGluR agonist DHPG (50 µM) facilitates the synaptic depression induced by bath applied NMDA (20 µM, for 3 minutes) to (56.0 ± 7.7% from baseline, n=13) as compared to control slices (74.6 ± 3.1% from baseline, n=11, $p < 0.05$). In the presence of ethanol (50 mM) and DHPG, throughout the recording sessions, the facilitation by DHPG was inhibited (depression of the fEPSP/PS amplitude to 80.2 ± 2.6% of baseline, n=10, $p < 0.05$) compared to the effect of NMDA in the presence of DHPG. b) Bar graph shows the averaged maximal effect of NMDA on fEPSP/PS amplitude measured in individual slices. NMDA induced synaptic depression is potentiated by DHPG. This effect of DHPG was reversed by ethanol. *$p < 0.05$.

It should be noted that the concentration of DHPG (50 µM) used in this study did not induce any significant depression in the fEPSP/PS amplitude by itself. Thus, these observations show that ethanol specifically inhibits the facilitation of NMDA-induced depression mediated by mGluR group I agonist. Our results are in line with the previous electrophysiological observations showing that ethanol may inhibit mGluR group I functions (Minami et al., 1998; Belmeguenai et al., 2008). Therefore, our data suggest a novel mechanism of action of acute ethanol in the NAc which involves mGluRs group I and their interaction with NMDA receptors. Importantly, it has been reported that when mGluR5 (group I) receptors in the NAc are blocked rats are unable to discriminate between alcohol and water i.e. mGluR5 receptors are required for the expression of the interoceptive effects of alcohol (Besheer et al., 2009) and for the maintenance of alcohol self-adminstration in alcohol-preferring rats (Besheer et al., 2010).

Although the above results suggest that ethanol disrupts an interaction between mGluR group I and NMDA receptors, this mechanism still does not fully explain how ethanol inhibits LTP. The experiments investigating the NMDA-induced synaptic depression were based on the assumption that activation of postsynaptic NMDA receptors would increase extracellular levels of adenosine which in turn would decrease glutamate release via stimulation of presynaptic adenosine A1 receptors (Schotanus et al 2006). To confirm this idea a paired-pulse stimulation protocol (20 ms interpulse interval) was used to identify a possible presynaptic locus of NMDA-mediated depressant action in a separate set of experiments. We found that 20 µM NMDA (bath applied for 3 minutes) decreased the amplitude of the first fEPSP/PS to 67.9 ± 4.0% of baseline (figure 11a) and increased the ratio between the second and the first fEPSP/PS from 1.0 ± 0.1 to 1.2 ± 0.1 (figure 11b).

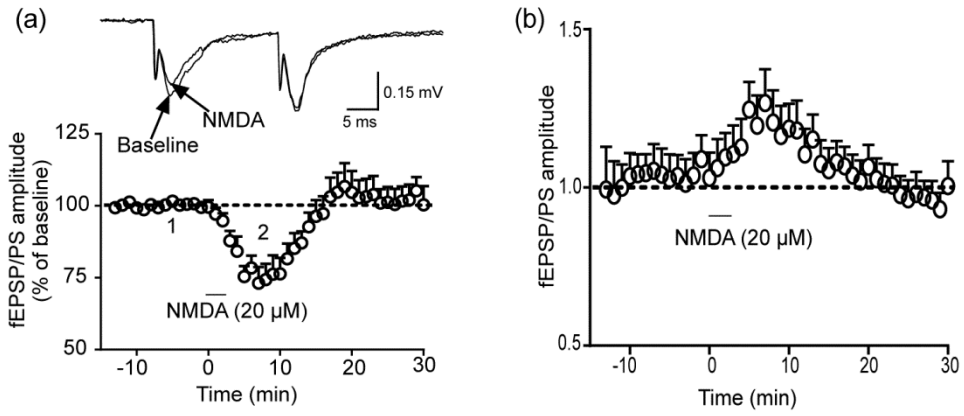

Figure 11. *N*-methyl-D-aspartate (NMDA) depresses glutamatergic synaptic transmission in the nucleus accumbens (NAc). a) NMDA (20 µM), applied for 3 minutes in the perfusion solution at the time indicated by the black bar, decreased the amplitude of the first field excitatory postsynaptic potential/population spike (fEPSP/PS) evoked by a paired-pulse stimulation protocol. Representative superimposed paired (20 ms apart) fEPSP/PSs, measured in the same slice before (baseline) and after NMDA are provided. b) Paired pulse ratio increased in (n = 8).

These results confirm that NMDA application decreases the probability of glutamate release from the presynaptic terminal, possibly via activation of presynaptic adenosine A1 receptors.

Given the result that ethanol application alone also decreased release-probability it may be inferred that the expected increase in release probability due to blockade of postsynaptic NMDA receptors by ethanol is masked by ethanol's presynaptic effect. In fact, ethanol has been shown to interact with the nucleoside transporter ENT1 in cultured cells and increase extracellular levels of adenosine (Nagy et al., 1990) and ENT1 null mice self-administer more ethanol than wild-type and have a higher alcohol preference (Choi et al. 2004).

4.4. Cortical glutamate levels in adolescent and adult animals (Paper III)

Using *in vitro* slice electrophysiology we found that acute ethanol inhibits the glutamatergic transmission in the NAc of adolescents more prominently than in adult animals. As PFC projects its glutamatergic glutamatergic input directly to NAc, the activity in PFC could potentially affect the glutamatergic transmission in the NAc (see Sesack and Grace, 2010). Therefore, to further understand the effect of acute ethanol on the glutamatergic system, we recorded glutamate release in the PFC of freely moving animals by using the newly designed glutamate-sensitive microelectrode array (MEA).

On experimental day 1 basal glutamate levels were determined, before saline was injected, in adolescent and adult animals based on their weights. Interestingly, we observed an age-dependent difference in cortical glutamate levels (figure 12a). The average basal glutamate levels in adolescent animals (7.7 µM ± 1.014, n=10) were more than three times higher compared to that of adult animals (1.7 µM ± 0.3, n=7, p <0.001, figure 12b).

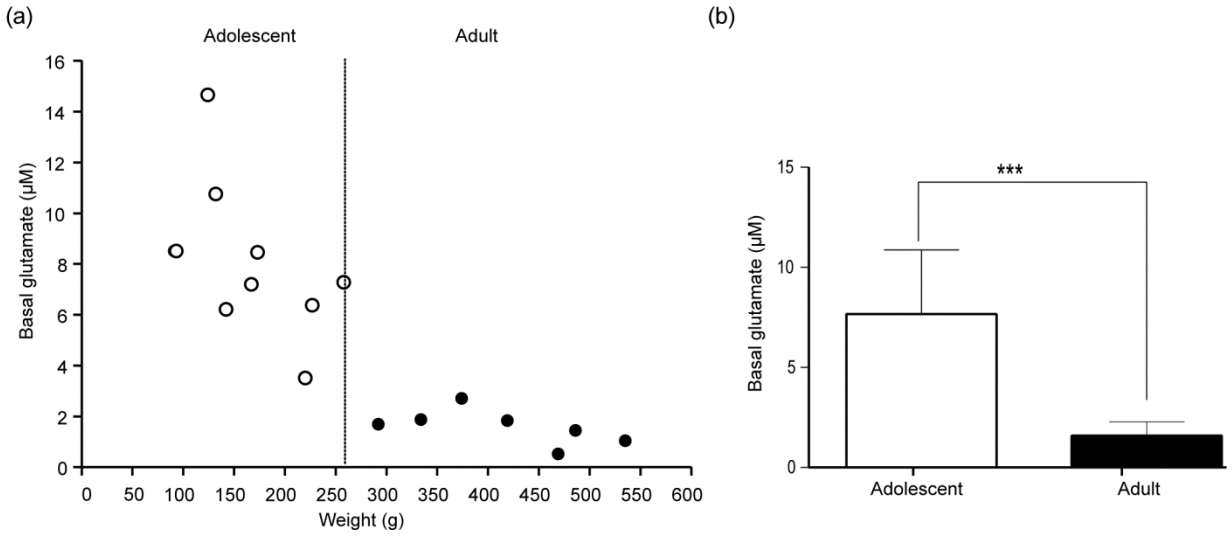

Figure 12. Basal cortical levels of glutamate on experimental day 1 in adolescent and adult animals. a) The basal glutamate levels decreased with weight (age), the cut off weight for adolescent animals was 260g. b) Adolescent animals showed more than three times higher cortical levels of glutamate (µM) compared to that of adults. Adolescent animals had significantly (p <0.001, using two-tailed Student's t-test, unpaired) higher basal levels of glutamate (7.7 µM ± 1, n=10) compared to adults (1.6 µM ± 0.3, n=6).

Based on my previous data and other studies using electrophysiology in brain slices showing that acute ethanol may inhibit glutamate release in many brain regions (e.g. NAc, hippocampus, basal amygdala; Nie et al., 1994; Steffensen et al., 2000; Hendricson et al., 2003; Roberto et al., 2004), I expected to see a decrease in glutamate levels after an acute ethanol injection. However, ethanol (1 g/kg) did not significantly affect basal glutamate levels in either age group during our experiments. This result was similar to a microdialysis study by Selim and Bradberry (1996) showing no effect of acute ethanol injections, at doses ranging from mild to heavily intoxicating (0.5 to 2.0 g/kg), on cortical glutamate levels in the PFC.

4.5. Glutamate transients and effects of ethanol injection (Paper III)

Glutamate transients are spontaneous glutamate peaks with a total duration of 1-3 seconds occurring with a variable frequency and amplitude which can be observed during recordings with MEA. Neither experimental day nor i.p. saline injection had any significant effect on the baseline frequency and amplitude of glutamate transients. Interestingly, the frequency and amplitudes of glutamate transients varied between adolescent and adult animals. The transient frequency was higher in adolescent rats (4.0 ± 1.0 /h) than in adults (0.5 ± 0.5 /h). This difference was statistically significant ($p < 0.03$). Also, the amplitude of glutamate transients was significantly higher in adolescent rats (3.5 ± 0.9 µM, n=8) than in adults (0.3 ± 0.3 µM, n=4, $p<0.05$) (data not shown).

Moreover, injection of ethanol (1 g/kg) in adolescent animals decreased the frequency (1.2 ± 0.6/hour, n=6) and the amplitude (1.9 ± 1.1 µM, n=6) of glutamate transients in the first hour when compared to transient frequency (4.0 ± 1.0 transients/hour, n=6) and amplitude (3.4 ± 1.2 µM, n = 6, $p < 0.01$) during the baseline (figure 13a and b). Additionally, in the same group of animals, during the third hour post-ethanol injection, we also observed an increased frequency (9.3 ± 2.3, n=6, $p<0.05$) of the transients. Transient amplitude was also slightly higher (5.3 ± 2.2 µM, n=6), but not significantly different. Ethanol injection in adult animals did not induce any significant changes in the frequency and amplitude of glutamate transients (figure not shown).

To further analyse the early inhibition and delayed potentiation of glutamate transients in the PFC induced by ethanol in adolescent animals, we compared the clearance rate (T80) of the transients before and after ethanol injection (figure 13c). We observed that the T80 values were not linearly proportional to the amplitudes of glutamate transients during baseline in adolescent rats (average T80=1.3 seconds ± 0.02, n=6) meaning that clearance of glutamate is not dependent on the amplitude of the glutamate transients. Following injection of ethanol, the clearance rate of glutamate seemed to be decreased during the first hour (2.1 seconds ± 0.5, n=4), when transient amplitude was decreased. This effect however, did not reach statistical significance. In the third hour following ethanol injection, the averaged T80 value decreased significantly (1.1 seconds ± 0.03, n=6, $p<0.01$) compared to baseline T80 values. This result indicates a faster clearance rate of glutamate during the third hour after ethanol injection when transient frequency was elevated.

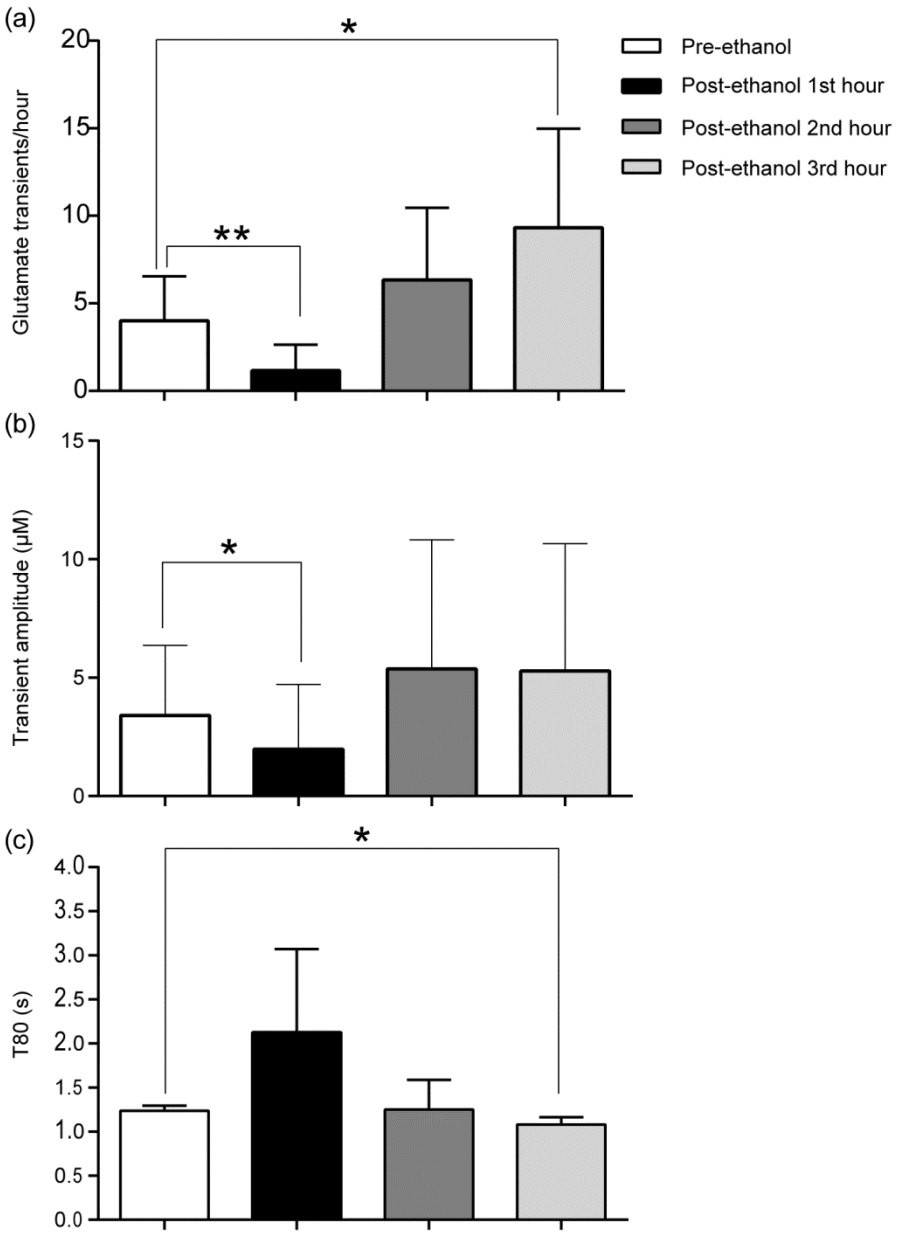

Figure 13. Effects of acute ethanol on glutamate transients in adolescent animals. a) Ethanol significantly inibited transient frequency (1.2 ± 0.6/hour, n=6, n=6 $p < 0.01$) in the first hour post-ethanol injection, followed by a rebound potentiation (9.3 ± 2.3, n=6, $p<0.05$) during the third hour post injection when compared to baseline (pre-alcohol) frequency (4.0 ± 1.0 transients/hour). b) Transient amplitude within the first hour post-ethanol (1.9 ± 1.1 µM, n=6, $p < 0.05$) was significantly decreased compared to baseline values (3.4 ± 1.2 µM, n = 6). Additionally, the transient amplitude within third hour post-ethanol was increased (5.3 ± 2.2 µM, n=6), but statistical significance (p=0.16) was not reached. c) The clearance rate (T80) in the first hour post-ethanol was increased (2.1 seconds ± 0.5, n=4), although, not significant (p=0.2) compared to clearance rate during baseline (1.3 seconds ± 0.0, n=6). During the third hour post-ethanol, the clearance rate was significantly decreased (1.0 seconds ± 0.0, n=6, $p<0.01$). All statistical comparisons were made using two-tailed Student's t-test for paired observations.

Glutamate transients have previously been reported by two other research groups (Hascup et al., 2011; Wassum et al., 2012) and the transient features seem to vary depending on the brain region studied and the experimental conditions, for example, we report a much lower transient frequency than both previous studies which in addition to differences between brain regions may also be related to the fact that we introduced R^2 correction (explained in methods, see

section 3.2.10.). Moreover, there has not been much research performed in order to understand the function or role of the glutamate transients. However, Wassum and colleagues (2012) studied glutamate transients in the BLA of rats that were trained to lever press to obtain a sucrose reward. In these experiments, glutamate transients preceded the learned lever-pressing behavior, without affecting the basal levels of glutamate, suggesting a functional importance of transients in the BLA. Moreover, infusion of the potent Na^+-channel inhibitor tetrodotoxin into the BLA in these rats, resulted in a decrease in transient frequency as well as a complete blockade of the behavior. Apparently, glutamate transients, rather than changes in basal levels of glutamate, may better reflect the importance of rapid glutamatergic synaptic processes.

5. General Discussion

The main findings of the present thesis are that acute ethanol inhibits glutamate release in the NAc, that this inhibition is stronger in adolescent animals than in adult animals and that ethanol inhibits LTP in this brain area. Moreover, glutamate levels in the PFC of adolescent animals are higher than in adults and ethanol inhibits glutamate release (transients) in adolescent animals.

It has been established that ethanol inhibits glutamate transmission by inhibiting NMDA receptors (see Lovinger and Roberto, 2013) but, as mentioned in the introduction, acute ethanol has also been suggested to inhibit glutamatergic transmission in adolescent and pre-adolescent animals via decreased glutamate release (Nie et al., 1994; Steffenson et al., 2000; Hendricson et al., 2003; Roberto et al., 2004). The data in the present thesis confirm that ethanol inhibits glutamate release in NAc of adolescent animals as well. Thus, ethanol was found to inhibit fEPSP/PS amplitudes more potently in adolescent animals than in adults. It should be mentioned that in most studies using in vitro electrophysiology, slices are prepared from young animals, typically at the age when the animals are in transition from adolescence to adulthood. Thereby, age-dependent differences may be overlooked. I found that the presynaptic inhibition of glutamate release induced by ethanol is dependent on both $GABA_A$ and $GABA_B$ receptor activation since it was blocked in the presence of antagonists at either receptor. This finding confirms previously published results (Steffensen et al. 2000) although that study did not compare with adult animals. Control experiments with GABA receptor agonists and antagonists applied alone indicate that the difference between adolescent and adult mice may in turn be related to differences in GABAergic neurotransmission since inhibitory tone appears to be higher in adult animals whereas GABA receptor function may be higher in adolescent animals. This age-dependent difference in GABAergic neurotransmission may be highly relevant for age-dependent behavioral differences in response to ethanol and should be investigated further, although that was not the focus of the present thesis.

In line with ethanol-induced inhibition of glutamate transmission it was found that ethanol inhibited the induction of LTP in the NAc. Surprisingly, ethanol's effect on glutamate release seemed unrelated to the mechanism by which ethanol inhibits LTP. LTP is dependent on postsynaptic NMDA receptor function and blockade of NMDA receptors prevents LTP, however, we were unable to see any interaction between ethanol (50 mM) and NMDA (bath applied) in the NAc. NMDA receptor stimulation has been suggested to increase adenosine release from the postsynaptic terminals, which in turn, decrease the release probability via stimulation of inhibitory adenosine A1 receptors present on the presynaptic terminals (Schotanus et al., 2006). As ethanol also inhibits NMDA receptors (see Lovinger and Roberto, 2013 and references therein), which in principle should increase release probability, this effect may be masked due to ethanol's interaction with other targets and the subsequent decrease in release probability. Clearly, multiple other factors related to the experimental conditions may also explain the

absence of an interaction between ethanol and NMDA receptors including animal species and age, the temperature during recordings, the presence or absence of Mg^{2+} ions in the perfusion solution and the concentrations of ethanol used (see Woodward et al., 2006). In addition, the effect of ethanol might also vary depending on the subunit composition of NMDA receptors (Izumi et al., 2005; Otton et al., 2009) which in turn varies with age of the animal and the brain region studied.

Instead, a novel interaction between mGluRs, NMDA receptors and ethanol was discovered. Activation of mGluRs by DHPG facilitated the NMDA-mediated response (synaptic depression) and this facilitation was prevented in the presence of ethanol. The fact that ethanol inhibited the facilitating effect of mGluR group I activation is rather unexpected considering the therapeutic potential of mGluR5 receptor antagonists and negative allosteric modulators at the mGluR5 receptor (see Olive, 2009 and Kalivas and Volkow, 2011). Thus, mGluR5 antagonism has been shown to prevent relapse to different drugs of abuse like cocaine and alcohol in animal models of addiction (Paterson et al., 2003; Kenny et al., 2003; McGeehan et al., 2004; Lee et al., 2005; Bäckström and Hyytiä, 2006; Besheer et al, 2006; Blednov et al., 2008). mGluR5 null mutant mice showed no cocaine-induced locomotor sensitization, they did not self-administer cocaine but still responded normally to natural rewards, (Chiamulera et al. 2001). Moreover, Besheer and colleagues (2009) injected mGluR5 antagonists in alcohol self-administering rats and found that it modulated the discriminative stimulus effect of alcohol and therefore, reduced the preference for alcohol in a two-bottle choice paradigm. Although, it is difficult to speculate on how my result, showing that ethanol is inhibiting mGluR5 function acutely, fits with the above mentioned studies, recent data actually suggest that positive allosteric modulation of mGluR5 may improve extinction learning (Gass and Olive, 2009; Cleva et al., 2011; Ben-Shahar et al., 2013) and there are also reports suggesting that the two members of the mGluR group I, mGluR1 and mGluR5 may exert opposing effects (Lominac, 2006; Liu et al. 2011). Needless to say, further research is required to understand the relevance of the herein reported novel interaction between ethanol and mGluR5 receptors for alcohol addiction.

Importantly, long term exposure to drugs of abuse followed by withdrawal has been associated with a loss of synaptic plasticity especially in the AcbC region (Martin et al., 2006; Kasanetz et al., 2010; Knackstedt et al., 2010: Gipson et al., 2013b) and this loss was specifically observed in animals that developed uncontrolled drug-intake and resistance to drug-associated negative consequences (Kasanetz et al., 2010; see review Kalivas and Volkow, 2011). Synaptic plasticity in the NAc, especially in the core region, is modulated by the prelimbic part of the PFC which sends glutamatergic inputs predominantly to the AcbC (Wright and Groenewegen, 1995; Groenewegen et al., 1999). In fact, Knackstedt and colleagues (2010) demonstrated that LTP in the NAc can be readily induced *in vivo* by stimulating the prelimbic afferents, but, after withdrawal from self- administration of cocaine LTP could no longer be induced following prelimbic stimulation. These data together with data from a vast number of studies point to the importance of the glutamatergic connection between the prelimbic part of the PFC and the

AcbC in addiction (see Gipson 2013a and references therein). Therefore, I continued to explore the effects of acute alcohol injection on glutamate release in the prelimbic/infralimbic region of adolescent and adult animals *in vivo*.

The basal level of cortical glutamate in adolescent PFC was more than three times that of adults. This result was intriguing, because during brain maturation the adolescent PFC has been proposed to be undergoing LTD majorly (see Selemon, 2013). Moreover, it is also known that the NMDA receptor density in an adolescent brain is higher compared to that of adult animals (Insel et al., 1990). Thus, the conditions in the adolescent PFC with high numbers of NMDA receptors and high levels of glutamate appear to favour potentiation or LTP, however, it is still not clear how this might be related to the process which is suggested to predominate during this age i.e. LTD and synaptic pruning (see Selemon 2013).

As mentioned above, the major form of plasticity occurring in the adolescent PFC has been suggested to be LTD which results from a weak afferent stimulation of a particular synapse, possibly leading to internalization of postsynaptic AMPA receptors (Malinow et al., 2002), and ultimately, elimination of the synapse i.e. synaptic pruning (Bear, 2003; Heynen et al., 2003). On the other hand, LTP is known to strengthen synaptic transmission via increased AMPA receptor expression on postsynaptic cells (Zhu et al., 2000; Sun et al., 2005; Makino et al., 2009) and therefore, a synapse undergoing LTP may escape synaptic pruning. Due to prevailing high extracellular basal levels of glutamate in adolescents, certain synapses may require stronger stimulation for strengthening and developing further. Based on the finding in the present thesis, that the frequency of glutamate transients was higher in adolescent than adult rats, it is tempting to speculate that glutamate transients play this significant role in adolescent brain during development.

Ethanol decreased both the frequency and amplitude of glutamate transients in adolescent animals. Our result thereby further supports the notion that ethanol inhibits glutamate release, particularly in adolescent animals. In the NAc the inhibition of glutamate release was blocked by a $GABA_B$ antagonist. It has previously been shown that ethanol´s inhibitory effect on glutamate release involves activation of presynaptic $GABA_B$ receptors (Steffensen et al. 2000). Ethanol has also been found to inhibit on L-type Ca^{2+}-channels on presynaptic terminals (Hendricson et al. 2003). As L-type Ca^{2+}-channels are targets of presynaptic $GABA_B$ receptors it is possible that this is a sequential mechanism by which ethanol inhibits glutamate release in the NAc and tempting to suggest that it is generalizable to other brain regions.

During the third hour after ethanol injection we observed a rebound potentiation of glutamate transients' frequency and amplitude associated with a faster glutamate clearance rate. LTP has been shown to be dependent on the reuptake rate of glutamate, i.e. faster glutamate reuptake supports both induction and maintenance of LTP (Pita-Almenar et al., 2006). Thus, ethanol-induced rebound potentiation of glutamate transients associated with a faster glutamate reuptake may contribute to induction and/or maintenance of LTP. In other words, inhibition of

glutamate transients acutely, followed by the delayed rebound potentiation, might trigger erroneous plasticity in postsynaptic neurons which may thereby result in incorrect pruning of synapses and/or synapses falsely preserved. Based on this reasoning, ethanol may not only inhibit the formation of normal synapses, but also recruit and strengthen some synapses that may provide susceptibility for further development of abuse and addiction.

Taken together, the present thesis demonstrates important age-dependent differences in the way ethanol affects glutamatergic neurotransmission and plasticity. As glutamatergic neurotransmission and plasticity are critically involved in brain maturation as well as in shaping addictive behavior the observations in the present thesis may constitute some of the underlying reasons for increased susceptibility of the adolescent individual to develop alcohol addiction later during life.

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